Remarks

Prior to this amendment, claims 1, 6-9, 11-15, 21, 25-28, 32-34, and 38-45 were pending in this application. Claims 10, 11, and 39 are amended, and claims 12 and 25 are canceled herein. Claim 10 is amended to correct a minor grammatical error and claim 11 is amended according to antecedent basis requirements. Support for the amendment of claim 39 can be found in the specification at page 22, lines 32-36.

No new matter has been added by these amendments. After entry of this amendment, **claims 1, 6-9, 11, 13-15, 21, 26-28, 32-34, and 38-45 are pending.** Unless specifically stated otherwise, none of these amendments are intended to limit the scope of any claim; Applicants reserve the right to pursue any removed subject matter in a related application.

Information Disclosure Statement

Applicants thank Examiner Huff for considering and initialing copies of the Information Disclosure Statements submitted on April 21, 2005 and March 13, 2006 (the latter received by the Patent Office on March 15, 2006).

Claim Rejections Under 35 U.S.C. §112, second paragraph

Claims 10-12, 26-28, and 39-45 are rejected under 35 U.S.C. 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants traverse these rejections; each rejection will be addressed separately, below.

Part (a)

The Office action does not explicitly state which claim is rejected for reciting "a[s] colon tumor a uterine tumor" in line 2. However, Applicants believe that this rejection is directed at claim 10 and will respond to the rejection based on this assumption. Claim 10 is amended to include a comma between "colon tumor" and "a uterine tumor." Applicants submit that this amendment renders claim 10 clear and definite and withdrawal of this rejection is respectfully requested.

Part (b)

Claims 11 and 12 are rejected for allegedly not having antecedent basis for "the agent." Claim 12 is canceled, rendering this rejection of claim 12 moot. Claim 11 is amended to recite "the monoclonal antibody." Applicants submit that the amended claim now properly depends from claim 1 and that this amendment renders this claim clear and definite. Withdrawal of this rejection of claim 11 is respectfully requested.

Part (c)

Claim 26 is rejected for allegedly not being clear as to what activity of the immune cell is enhanced. Applicants respectfully disagree with the Office action. Claim 26 clearly recites that "blocking the TGF-β signaling pathway results in *increased tumor immunosurveillance* by the TGF-β receptor-expressing cell" (emphasis added). Furthermore, the specification clearly teaches that an enhanced activity of an immune cell, resulting from the administration of an agent which blocks the TGF-β signaling pathway, is *increased tumor immunosurveillance* (see specification at least at page 22, lines 1-4). Thus, the enhanced activity of the immune cell is *increased tumor immunosurveillance*. In light of the above discussion, Applicants submit that claim 26 is clear and definite and withdrawal of the rejection is respectfully requested.

Part (d)

Claim 39 is rejected for allegedly not being clear as to whether the TGF-beta cell in line 3 is the same as the control TGF-beta cell in lines 6 and 7. Applicants respectfully disagree with the Office action. However, solely to advance prosecution in this case, claim 39 is amended to recite ". . . a TGF-β receptor-expressing control immune cell of the same type not contacted with the agent, and wherein the decrease in activity of TGF-β signaling in the TGF-β receptor-expressing immune cell is indicative of an agent that inhibits tumor recurrence in a subject." Applicants submit that this amendment renders claim 39 clear and definite and withdrawal of this rejection is respectfully requested.

Claim Rejections Under 35 U.S.C. §112, first paragraph (enablement)

Claims 39-45 are rejected as allegedly failing to comply with the enablement requirement. Applicants traverse this rejection of claims 39-45.

The Federal Circuit has repeatedly stated that enablement is not precluded by the necessity for some experimentation, so long as the experimentation is not undue. *In re Wands* 8 USPQ2d 1400 (Fed. Cir. 1988). A considerable amount of experimentation is permissible, if it is **merely routine**, or if the specification provides a reasonable amount of guidance in which direction the experimentation should proceed. *Id.* Applicants submit that any experimentation would be routine and the present application provides the guidance necessary to understand and carry out the methods encompassed by the claims.

Claims 39-45 are directed to a method for screening for an agent that inhibits tumor recurrence. Applicants submit that representative candidate agents that can be used to block the TGF-beta signaling pathway and inhibit tumor recurrence are clearly described in the specification (page 22, line 31 through page 24, line 20). In addition, the specification discloses example assays to use in order to measure the effect of such agents on the TGF-beta signaling pathway (page 24, line 23 through page 25, line 5). The specification also clearly sets out various methods for identifying which agents can inhibit tumor recurrence (page 26, line 5 through page 27, line 18). Finally, the specification teaches how to use agents once they have been identified as blocking the TGF-beta signaling pathway in order to inhibit tumor recurrence. For example, the specification teaches routes of administration of an agent to a subject and timing of administration of an agent.

Thus, Applicants contend that the specification provides sufficient guidance for one of skill in the art to understand and perform the claimed screening methods in order to identify an agent that can inhibit tumor recurrence. Further, at the time the application was filed it was well known to those of skill in the art how to use *in vitro* assays to test the effectiveness of various agents for clinical use. It was also well known at the time how to test promising candidate agents *in vivo* using any one of a number of animal models. Thus, Applicants submit that, given the state of the art at the time of filing and the guidance in the specification, it would merely be **routine** to perform the method of screening for an agent that inhibits tumor recurrence, particularly in view of Applicants' specific teachings in the specification.

In light of the above arguments, Applicants submit that claims 39-45 are fully enabled by the specification. Applicants request that the rejection under 35 U.S.C. §112, first paragraph, be withdrawn.

Claim Objections

Claim 12 is objected to as allegedly failing to further limit the subject matter of a previous claim. Solely to advance prosecution in this case, claim 12 is canceled, rendering this rejection of claim 12 moot.

Double Patenting

Claim 25 is provisionally objected to as allegedly being a substantial duplicate of claim 1. Applicants traverse this objection. However, solely to advance prosecution in this case, claim 25 is canceled, rendering this provisional objection moot.

Claim Rejections Under 35 U.S.C. §102(b)

Claims 1, 6-9, 11-15, 21, 25-28, 32-34, and 38 are rejected under 35 U.S.C. §102(b) as allegedly anticipated by Dasch *et al.* (U.S. Patent No. 9,090,383) because the reference "discloses and claims methods for treating tumor cells by administering monoclonal antibodies reactive to TGF-beta and to permit generation of an immune response against the tumor and this results in tumor regression" (Office action at page 6). Applicants strenuously traverse this rejection. Claims 12 and 25 are canceled, rendering the rejection of these claims moot.

Applicants note that the specification clearly discusses recognized distinctions among original (primary) tumors, recurrent tumors, and metastases (secondary) tumors. A tumor recurrence is the "return of a tumor, at the same site as the original (primary) tumor, after the tumor has been removed surgically, by drug or other treatment, or has otherwise disappeared" (page 17, lines 23-25). A tumor recurrence is not a metastasis, as a metastasis is the "spread of a tumor from one part of the body to another. Tumors formed from cells that have spread are called "secondary tumors" and contain cells that are like those in the original (primary) tumor" (see specification at page 10, lines 25-29). It was well known in the art at the time the application was filed that there can be a recurrence of either a primary tumor or a metastasis.

However, cells of a recurrent primary tumor or a recurrent metastatic tumor differ immunologically from the original primary tumor or original metastatic tumor. As a result, a recurrent tumor, which appears following treatment to induce regression and/or disappearance of a primary (original) tumor, is insensitive to the treatment used to regress the original tumor. For example, treatments that effectively regress a primary tumor have been shown to be ineffective at subsequently inhibiting a recurrent tumor (Restifo *et al., J. Natl. Can. Inst.*, 88:100-108, 1996 – Exhibit A; Maeurer *et al., J. Clin. Invest.*, 98 :1633-1641, 1996 – Exhibit B ; Lee *et al., J. Immunol.*, 161:4183-4194, 1998 – Exhibit C; Matusi *et al., J. Immunol.*, 163:184-193, 1999 – Exhibit D). In fact, this phenomenon continues to be an active topic of scientific investigation (Khong *et al., J. Immunother.*, 27:184-190, 2004 – Exhibit E; Kmieciak *et al., Eur. J. Immunol.*, 37:675-685, 2007 – Exhibit F). Thus, the characteristics of a recurrent tumor (or recurrent metastatic tumor) are very different from the original primary tumor (or original metastatic tumor), despite the fact that the recurrent tumor exists at the site of the original tumor. Based on what was known in the art at the time the application was filed, one of ordinary skill would not have expected or predicted that an antibody used to treat a primary tumor would be effective at treating a tumor recurrence.

As discussed above, Dasch *et al.* discloses the use of a TGF-beta antagonist (the 1D11.16 monoclonal antibody) to *regress existing tumors* (column 5, lines 54-58) or to treat tumors cells that produce TGF-beta (column 2, lines 28-32). Applicants note that inducing the regression of a tumor is very different than inhibiting recurrence of a tumor, as the former reduces the size of an existing tumor and the latter prevents the return of a variant form of the tumor. Applicants also note that Dasch *et al.* does not specifically teach the use of the disclosed TGF-beta antagonists to *inhibit the recurrence* of a tumor that has escaped tumor immunosurveillance or to inhibit a variant of a tumor that produces TGF-beta. In this regard, Dasch *et al.* does not disclose the concept of tumor recurrence or the modified characteristics of tumor cells which make them resistant to the treatment which was effective against the original tumor. Nor would it be known from the teachings of Dasch *et al.* that the disclosed TGF-beta antagonists could inhibit tumor recurrence. Thus, Dasch *et al.* does not and cannot anticipate Applicants' claims. Based on the above discussion, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 6-9, 11-15, 21, 25-28, 32-34, and 38.

Claim Rejections Under 35 U.S.C. §103(a)

Dasch *et al.* in view of Suthanthiran *et al.*

Claims 1, 6-15, 21, 25-28, 32-34, and 38 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Dasch *et al.* in view of Suthanthiran *et al.* (U.S. Patent Application No. US2004/0197333). As discussed above, Dasch *et al.* does not specifically teach the use of the disclosed TGF-beta antagonists to inhibit the recurrence of a tumor that has escaped tumor immunosurveillance. Suthanthiran *et al.* does not make up for this deficiency.

Suthanthiran *et al.* discloses methods of "reducing formation or progression of a neoplasm associated with immunosuppressive therapy in a mammal by treating with a TGF-beta antagonist" ([0017]). However, Suthanthiran *et al.* defines "reducing formation or progression of a neoplasm" as "prevention or inhibition of initiation, establishment, proliferation or metastasis of the neoplasm" ([0017]). Suthanthiran *et al.* does not teach the use of TGF-beta antagonists to *inhibit the recurrence* of a tumor that has escaped tumor immunosurveillance. Nor does Suthanthiran *et al.* disclose the concept of tumor recurrence or the modified characteristics of tumor cells which make them resistant to the treatment which was effective against the original tumor.

Because neither Dasch *et al.* nor Suthanthiran *et al.* implicitly or explicitly teach all elements of the claimed methods, Applicants' claims are non-obvious over the cited references. Withdrawal of this rejection is requested.

Dasch *et al.* in view of Terabe *et al.*

Claims 1, 6-15, 21, 25-28, 32-34, and 38 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Dasch *et al.* in view of Terabe *et al.* (*Nature Immunol.*, 1:515, 2000). The Office action states that Dasch *et al.* "discloses the use of the mab in an assay to monitor tumor mass (col. 6, lines 44-61)" (Office action at page 10). Applicants note that the cited passage in Dasch *et al.* does not refer to monitoring tumor mass. Instead, col. 6, lines 44-61 refers to the use of the disclosed antibody in "a number of immunoassays, including assays to

purify or quantify TGF-B." The claims are directed to a method of *inhibiting tumor recurrence* using a monoclonal antibody specific for TGF-beta. As this passage does not relate to the use of the antibody to inhibit tumor recurrence, it is not relevant to this rejection. The Office action also states that as Dasch *et al.* discloses methods for monitoring tumor progression, this reads on tumor immunosurveillance. Applicants submit that monitoring tumor progression does not encompass inhibiting tumor recurrence.

As discussed above, Dasch *et al.* does not teach the use of the disclosed TGF-beta antagonists to *inhibit the recurrence of a tumor* that has escaped tumor immunosurveillance. Terabe *et al.* does not make up for this deficiency. Terabe *et al.* discloses that natural killer T cells and interleukin-13 are necessary for down-regulation of tumor immunosurveillance. Terabe *et al.* also discloses various assays that measure tumor immunosurveillance. However, Terabe *et al.* does not teach the use of the disclosed TGF-beta antagonists to *inhibit the recurrence of a tumor* that has escaped tumor immunosurveillance.

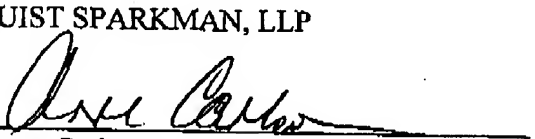
Because neither Dasch *et al.* nor Terabe *et al.* implicitly or explicitly teach all elements of the claimed methods, these claims are clearly non-obvious over the cited references. Withdrawal of this rejection is requested.

Conclusion

Based on the foregoing amendments and arguments, the claims are in condition for allowance and notification to this effect is requested. If for any reason the Examiner believes that a telephone conference would expedite allowance of the claims, please telephone the undersigned at the number listed below.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

By 

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Notes

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The Netherlands Cohort Study on Diet and Cancer was approved by the Review Boards of the TNO Nutrition and Food Research Institute and of the University of Limburg.

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Loss of Functional Beta$_2$-Microglobulin in Metastatic Melanomas From Five Patients Receiving Immunotherapy

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Background: **In a subset of patients with metastatic melanoma, T lymphocytes bearing the cell-surface marker CD8 (CD8$^+$ T cells) can cause the regression of even large tumors. These antitumor CD8$^+$ T cells recognize peptide antigens presented on the surface of tumor cells by major histocompatibility complex (MHC) class I molecules. The MHC class I molecule is a heterodimer composed of an integral membrane glycoprotein designated the α chain and a noncovalently associated, soluble protein called beta$_2$-microglobulin (β_2m). Loss of β_2m generally eliminates antigen recognition by antitumor CD8+ T cells. *Purpose:* We studied the loss of β_2m as a potential means of tumor escape from immune recognition in a cohort of patients receiving immunotherapy. *Methods:* We successfully grew 13 independent tumor cell cultures from tumor specimens obtained from 13 patients in a cohort of 40 consecutive patients undergoing immunotherapy for metastatic melanoma and for whom tumor specimens were available. These cell lines, as well as another melanoma cell line (called 1074mel) that had been derived from tumor obtained from a patient in a cytokine–gene therapy study, were characterized in vitro cytofluorometrically for MHC class I expression and by northern and western blot analyses for messenger RNA (mRNA) and protein expression, respectively, and ex vivo by immunohistochemistry. *Results:* After one melanoma cell line (1074mel) was found not to express functional β_2m by cytofluorometric analysis, four (31%) of the 13 newly established melanoma cell lines were found to have an absolute lack of functional MHC class I expression. Northern blot analysis of RNA extracted from the five cell lines exhibiting no functional MHC class I expression showed that these cells contained normal levels of α-chain mRNA but variable levels of β_2m mRNA. In addition, no immunoreactive β_2m protein was detected by western blot analysis. When human β_2m was transiently expressed with the use of a recombinant vaccinia virus, cell-surface MHC class I expression was reconstituted and the ability of these five cell lines to present endogenous antigens was restored. Immunohistochemical staining of tumor sections revealed a lack of immunoreactive MHC class I in vivo, supporting the notion that the in vitro observations were not artifactual. Furthermore, archival tumor sections obtained from patients prior to immunotherapy were available from three patients and were found to be β_2m positive. This result was consistent with the hypothesis that loss of β_2m resulted from immunotherapy. *Conclusions:* These data suggest that the loss of β_2m may be a mechanism whereby tumor cells can acquire immunoresistance. This study represents the first characterization of a molecular route of escape of tumors from immune recognition in a cohort of patients being treated with immunotherapy. [J Natl Cancer Inst 1996;88:100-8]**

A subset of T lymphocytes bearing the cell-surface marker CD8 can be shown to directly lyse tumor cells in vitro (*1,2*). These T cells, designated CD8$^+$ T cells, can be expanded to large numbers ex vivo and adoptively transferred back to patients together with interleukin 2 (IL-2) where they can, in some cases, effect the regression of even large tumors (*3-5*). In patients with a number of different human malignancies, antitumor T cells can be elicited that are capable of recognizing autologous tumor cells as measured by cytolytic- and cytokine-

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See "Notes" section following "References."

release assays (*6-10*). Tumor deposits from patients with metastatic melanoma lesions yield tumor-infiltrating lymphocytes (TILs), with antitumor specificity in approximately 30% of the cases, making melanoma the most tractable of human cancers to T-cell-based immunotherapy. Objective clinical responses are observed in approximately 35% of patients with metastatic melanoma who are treated with TIL-based therapy; some responses are complete and long lasting [reviewed in (*11*)].

The reasons why the metastatic lesions of some patients yield successful TIL cultures ex vivo while others do not are unknown. It is also unknown why some patients with apparently specific and lytic TILs often fail to respond. Even more perplexing is why some tumors escape after an initial response to therapy and why only some, but not all, of the lesions in an individual respond to treatment.

Abnormalities in T-cell signal transduction induced by tumor cells are potential mechanisms for tumor escape from immune recognition (*12*). However, since most patients with melanoma do not have measurable immunosuppression, the tumor cell has become the target of investigation to determine the mechanisms of escape from immunologic recognition (*13*). The steps required for the processing and presentation of antigens for recognition by $CD8^+$ T cells, in particular, may be involved in tumor escape, since $CD8^+$ T cells do not recognize intact antigens on the surfaces of tumor cells but generally recognize peptide fragments of protein antigens presented by major histocompatibility complex (MHC) class I molecules (*14-16*).

MHC molecules are known as human leukocyte antigens (HLAs) in humans and H-2 antigens in mice (*17*). MHC class I molecules are heterodimers composed of a 44- to 46-kd integral membrane glycoprotein designated the α chain and a noncovalently associated, soluble 12-kd protein called beta$_2$-microglobulin (β_2m). All nucleated cells in the body, with the exception of germline cells and some neurons (*18*), express class I–peptide complexes, the ligands for CD8+ T cells. A molecular understanding of the structure of MHC molecules has made clear their true function with respect to antigen recognition by T cells: MHC molecules are receptors for peptide antigens (*19*). MHC molecules are physically associated with peptide antigens, and x-ray crystallographic data have indicated precisely how such an interaction occurs. The solution of the crystal structure of MHC class I and II molecules has clarified the molecular structure of MHC molecules and specifically the way MHC molecules bind antigen (*20-22*). MHC molecules have peptide-binding domains, consisting of a deep groove that runs between two long α helices found on the outward-facing surface of the MHC molecule. In the case of class I molecules, x-ray crystallographic findings have since been refined and extended to include x-ray images of particular peptides lying in the cleft of MHC molecules (*23-26*). These findings reveal that the peptides are bound in an extended conformation. Antigenic peptides bound by MHC class I molecules are generally eight to 10 amino acids in length, usually resulting from proteolytic activity in the cytoplasm.

Peptides are transported into the endoplasmic reticulum by a specialized adenosine triphosphate-dependent transporter called TAP, which is related to the multidrug resistance protein, Mdr1. Peptides then assemble together with α chains and β_2m to form a trimolecular complex that is then transported out of the endoplasmic reticulum, through the Golgi apparatus, to the cell surface for potential recognition by CD8+ T cells. With few exceptions (*27*), recognition by $CD8^+$ T cells does not occur in the absence of β_2m (*28,29*), since MHC class I α chains not associated with β_2m are retained in the endoplasmic reticulum (*30*).

Bicknell et al. (*31*), Bodmer et al. (*32*), and Momburg and Koch (*33*) have shown that human cancer cells, especially cells of adenocarcinomas of the colon, may fail to express β_2m; Wang et al. (*34*) and D'Urso et al. (*35*) have shown that two human melanoma cell lines do not express MHC class I as a result of mutations in the genes encoding β_2m. The loss or mutation of the genes encoding β_2m is a highly efficient mechanism for tumor escape from immune recognition, since stable presentation of peptide antigen by MHC class I molecules does not occur in the absence of β_2m. The purpose of our study was to investigate the loss of β_2m as a potential means of tumor escape from immune recognition in a cohort of patients undergoing immunotherapy.

Materials and Methods

Patients

Tumors were obtained according to institutional guidelines, and National Institutes of Health (NIH) Clinical Center standards for informed consent were adhered to for all patients. Thirteen independent tumor cell cultures were successfully grown from a cohort of 40 consecutive patients receiving immunotherapy for whom tumor specimens were available. These patients were enrolled in clinical trials at the National Cancer Institute (NCI), Bethesda, MD; the results of one of these trials was recently published (*4*).

Tumor Cells

Melanoma cell lines (1074mel, 1106mel, 1180mel, 1174mel, 1259mel, 526mel, 888mel, 397mel, and 624mel) were established in our laboratory, and the last four of these cell lines have been previously described (*36*). One tumor cell culture (1074mel; i.e., melanoma cell culture from patient No. 1074) was not part of the cohort of patients undergoing immunotherapy for metastatic melanoma and was derived for a different purpose [a cytokine-gene-therapy study (*37*)]. The β_2m-deficient melanoma cell line FO-1 has been previously described (*35*). Daudi (a B-lymphoblastoid cell line derived from a patient with Burkitt's lymphoma), H82sclc [a small-cell lung cancer cell line (*14*)], and K562 (human chronic myelogenous leukemia) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Tumor cells were maintained as a monolayer culture in complete medium containing RPMI-1640, 10% heat-inactivated fetal calf serum (both from Biofluids, Rockville, MD), and 0.03% (100 m*M*) glutamine (NIH Media Unit, Bethesda, MD).

Cytofluorography

Cultured tumor cell lines were harvested with 0.05% trypsin–0.02% versene without calcium and magnesium (Biofluids), washed, then incubated with hybridoma culture supernatant containing the W6/32 monoclonal antibody (MAb) for 30 minutes (Sera Labs, Westbury, NY) (mouse immunoglobulin G2a [IgG2a] isotype). W6/32 reacts with monomorphic determinants on the HLA-A, -B, and -C molecules. In all cases, cells were stained with the appropriate isotype-matched control antibody, nonspecific mouse IgG2a (Becton Dickinson Immunocytometry Systems, San Jose, CA). MAb binding to cells was followed by binding with goat anti-mouse fluorescein isothiocyanate-conjugated antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) and detected by fluorescence-activated cell sorting (FACS) using a FACScan 440 (Becton Dickinson, Mountain View, CA).

Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes (CTLs) were generated from excised tumor specimens by culturing suspension cells in complete medium containing IL-2

(6000 U/mL) (Chiron Therapeutics, Emeryville, CA) for 30-70 days as previously described (*38*).

Experiments that use mouse CTLs to study antigen processing in human tumors have been described elsewhere (*14*). In brief, polyclonal $CD8^+$ T-cell populations were generated from 6- to 8-week-old female BALB/c mice by intravenous injection of 5×10^6 plaque-forming units of vaccinia virus. After at least 2 weeks, spleens were removed, dispersed to single-cell suspensions, and stimulated in vitro with vaccinia-infected BALB/c splenocytes at a ratio of 2:1. Cells were then cultured in complete medium to generate $CD8^+$ T cells. The care of animals was in accord with NIH guidelines.

Microcytotoxicity Assays

^{51}Cr release assays were performed as previously described (*39*). Briefly, 5000 target cells labeled with 200 μCi of $Na^{51}CrO_4$ (Du Pont NEN, Boston, MA) were mixed with various numbers of effector cells and incubated for 6 hours. In experiments involving infection of cells with vaccinia virus, target cells were infected with 10 plaque-forming units per cell of the designated recombinant vaccinia virus(es) for 60-90 minutes, then labeled with ^{51}Cr for 1 hour. Target cells were then washed three times and mixed with $CD8^+$ T cells. In all microcytotoxicity assays, the amount of released ^{51}Cr was determined by γ-photon counting of aliquots of supernatants of cells after lysis experiments. Percent specific lysis was calculated as follows: ([experimental cpm–spontaneous cpm]/[maximal cpm–spontaneous cpm]) × 100.

Immunohistochemical Staining of Archival Tissue Specimens From Patients

Immunohistochemistry was performed on archival tissue sections where available. Formalin-fixed, paraffin-embedded sections, 5 μm thick, were deparaffinized in xylene and stained as previously described (*40*). Murine MAb, ATCC HB159 (anti-human β_2m, also known as SF1-1.1.1), supernatant was brought to pH 7 by the addition of HCl and used at a 1:5 dilution in phosphate-buffered saline (PBS) (Biofluids). Biotinylated horse anti-mouse IgG at 1:200 was used as the secondary antibody (Boehringer Mannheim Biochemicals). Immunoperoxidase staining was completed with the use of the avidin–peroxidase complex (Vector Laboratories, Inc., Burlingame, CA). Diaminobenzidine-hydrogen peroxide (Sigma Chemical Co., St. Louis, MO) was used as the developing solution, and the sections were counterstained with hematoxylin–eosin.

Western Blot Analyses

Tumor cells were lysed by adding a buffer consisting of PBS containing 2 m*M* EDTA, 2 m*M* phenylmethylsulfonyl fluoride, and 1 m*M* dithiothreitol (Sigma Chemical Co.) at 4 °C. The cells were then sonicated for 10-15 seconds, and the lysate was centrifuged at 3000 rpm for 5 minutes at 4 °C. The concentration of protein in the cell homogenate supernatant was determined by the use of the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Equal amounts of protein (20 μg) from each cell lysate were electrophoretically resolved on a 16% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane with the use of a Bio-Rad Transblott Mini cell (Bio-Rad) at 250 mA for 1 hour. The nitrocellulose sheet was blocked overnight with Blocking Buffer (5 Prime → 3 Prime, Inc., West Chester, PA) and first incubated with the anti-β_2m MAb HB159 (ATCC) for 2 hours and then with horseradish peroxidase-labeled secondary goat–anti-mouse antibody for 2 hours. Immunofluorescence was detected with the use of the Enhanced Chemi Luminescence (Amersham Life Science Inc., Arlington Heights, IL). Protein sizes were estimated with the use of low-molecular-weight standards (14 000-70 000; Sigma Chemical Co.).

Northern Blot Analysis

Specific probes for β_2m were generated by the use of the reverse transcription–polymerase chain reaction (PCR) method. The total RNA was isolated with the use of guanidine isothiocyanate (Fluka Chemical Corp., Ronkonkoma, NY) then purified with the use of the cesium chloride (Serva Biochemicals) centrifugation method, as previously described (*14*) from the Epstein-Barr virus-transformed B-cell line designated 501 that was established in our laboratory (*41*). First-strand complementary DNA (cDNA) was synthesized from 10 μg total RNA using an oligo dT primer and murine leukemia virus reverse transcriptase (Life Technologies [GIBCO BRL], Gaithersburg, MD). The specific 5′-side oligonucleotide primer for β_2m that was used had the sequence 5′ CAC GTC ATC CAG AGA ATG G 3′, and the 3′-side oligonucleotide primer had the sequence 5′ CGA TCC CAC TTA ACT ATC TTG G 3′. For PCR amplification, samples were initially denatured for 2 minutes at 94 °C, then 30 cycles were performed with the use of the following conditions: 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute followed by an extension cycle of 10 minutes at 72 °C. PCR products were electrophoresed on 1.5% agarose gels with an acetate buffer, and the predicted fragment sizes of bands (260 bp) were isolated, purified by the glass-powder method (GeneClean, La Jolla, CA), and used as hybridization probes for northern blots. The HLA-A2.1 full-length cDNA was used as a probe template for MHC class I (*42*). Human β-actin cDNA was purchased from Clontech Laboratories, Inc., Palo Alto, CA. The probes were labeled with [^{32}P]-deoxycytidine triphosphate (Amersham Life Science Inc.) by the random-priming method as previously described (*14*). For the northern blot analysis, 10 μg of total RNA from each tumor was electrophoresed in a 1% agarose–formaldehyde gel, transferred to a nylon membrane (Zeta-Probe, Bio-Rad Laboratories), and fixed to the membrane by UV cross-linking. Prehybridization was done for 30 minutes with the use of the 5 Prime → 3 Prime Northern Hybridization buffer. Hybridization was done in a 40% formamide hybridization solution (Northern hybridization buffer, 5 Prime → 3 Prime, Inc.) at 42 °C overnight. Membranes were washed three to four times in 2× standard saline citrate at 60 °C for 30 minutes for each wash, and autoradiography was then performed.

Gene Replacement Studies

The production of a recombinant vaccinia virus expressing the K^d gene (K^d-Vac) has been described (*14*), as has the production of the recombinant vaccinia virus containing the β_2m gene (β_2m-Vac) (*42*).

Results

Steady-state cell surface expression of MHC class I was studied in a group of patients with metastatic melanoma at the NCI. All of the patients entered in the T-cell-based immunotherapy protocol had either failed standard therapy or had disease for which no effective therapy was available. All patients had clinically assessable disease and received no other therapy during the 30 days before the protocol treatment or during treatment. Patient characteristics, including the immunotherapy received, are shown in Table 1.

Of the 40 attempts to grow tumor cells obtained from patients from whom melanoma deposits were surgically resected, 18 tumor cell lines could not be grown at all and nine could be derived, but did not grow adequately, and therefore were not studied for their expression of cell surface HLA. Of the 13 tumor cell lines that could be derived into tumor cell cultures that could be grown to quantities sufficient for in vitro evaluation, four were found to be devoid of MHC class I and were included in the present study for the characterization of their MHC class I deficits. One additional tumor cell culture (from patient No. 1074) was not part of this group of 40 and was derived for a different purpose [a cytokine gene-therapy study (*37*)], but is nevertheless included here because it was observed to be MHC class I deficient.

From this screening analysis, five independently established melanoma cell cultures from patients with metastatic melanoma were devoid of cell-surface HLA class I molecules as measured cytofluorometrically (Fig. 1). The loss of MHC class I expression on these cell lines was complete, even when assessed by the MAb W6/32 (that can recognize virtually all human HLA class I alleles).

Table 1. Patient characteristics

Patient No.	Age, y; sex	Specimen site*	Comments†	Tumor-infiltrating lymphocyte growth	Class I, histology	Response to immunotherapy‡
1106	46; male	SC	Previous response to treatment with IL-2 and IFN α; recurred and did not rerespond	No	No	—
		SC	Same as above	Yes	Yes§	Mixed
		SCII	Treatment with TILs from above, no response	No	No¶,#	—
		SC	Additional biopsies	No attempt	No	—
1180	46; female	SC	No previous treatment of metastasis	Yes	Yes	No
		SC	New nodule after no response to treatment with TILs/IL-2/IFN α	Yes	Low**	No
		SCII	New nodules after no response to TILs/IL-2/IFN α	No	No††,‡‡	—
1074§§	26; female	SCII	Previous mixed response to IL-2	Yes	No	No
1174	53; male	LNII	No response to previous IL-2	No	No	—
1259	30; male	SC	No previous immunotherapy	Yes	Yes	Yes
		SCII	Previous response to TILs; given cryopreserved TILs	No	No	No

*SC = subcutaneous; LN = lymph node.
†IL-2 = interleukin 2; IFN α = interferon alpha; TILs = tumor-infiltrating lymphocytes.
‡For definitions of clinical responses, *see* (*4*).
§Immunohistochemical staining, shown in Fig. 6, panel B.
¶Tumor from which melanoma cell line was derived.
‖Hematoxylin–eosin stain, shown in Fig. 6, panel A.
#Immunohistochemical staining, shown in Fig. 6, panel C.
**Immunohistochemical staining, shown in Fig. 6, panel E.
††Hematoxylin–eosin stain, shown in Fig. 6, panel D.
‡‡Immunohistochemical staining, shown in Fig. 6, panel F.
§§From another study (*42*).

To characterize the mechanisms underlying the lack of MHC class I expression, we considered the possibility that HLA α chains were poorly loaded with peptides in the endoplasmic reticulum because of down-regulation or mutation of the TAP peptide transporters or proteasome components, since we have observed this mechanism to be functioning in small-cell lung cancer (*14*). However, defects in the above-named components of the antigen-processing machinery have generally resulted in MHC class I expression that is decreased, but not absent, since some peptides can originate from peptides or proteins translocated across the endoplasmic reticulum membrane by TAP-independent signal sequence mechanisms, exposing the proteins to proteolytic activities in the endoplasmic reticulum (*43,44*).

The completeness of the loss observed in the melanoma cell lines suggested the structural loss or mutation of either all of the heavy-chain MHC class I molecules or of β_2m. The former would require disabling mutations of six independent genomic transcripts (two alleles each of HLA-A, -B, and -C) or the complete loss of approximately 1500 bp of the MHC class I region on the short arm of both copies of chromosome 6 (since MHC class I molecules are codominantly expressed). Another, more likely, possibility was the loss or mutation of both copies of β_2m, a structural component of cell-surface MHC class I.

To explore the possibility that the deficit in functional β_2m occurred at the level of decreased gene transcription or increased degradation of messenger RNA (mRNA), northern blot analysis of the melanoma tumor cell lines was performed. The α-chain mRNA levels were found to be normal (data not shown). However, expression of β_2m mRNA was variable and lower than that seen in control cell lines (Fig. 2). The controls used in this experiment were the antigen-presentation normal melanoma cell lines 624 and 397 and the FO-1 cell line, which is known to have a defect in the expression of functional β_2m, due to a lack of β_2m gene expression (*35*). A western blot analysis, however, failed to detect any immunoreactive protein (Fig. 3).

To test the hypothesis that β_2m was responsible for the absence of MHC class I on the cell surfaces, we sought to replace functional β_2m within the cells. Since MHC class I molecules are generally retained in the endoplasmic reticulum if they are not complexed with β_2m, the addition of functional β_2m would likely reconstitute cell-membrane expression of MHC class I molecules. One rapid, although transient, method of β_2m gene expression involved infection of the cells with a recombinant vaccinia virus that expresses β_2m. As shown in Fig. 1, expression of β_2m mRNA by a recombinant vaccinia virus elicited MHC class I expression on the surfaces of the five human melanoma cell lines under study. Indeed, there may be some suppression of endogenous MHC class I expression by control vaccinia virus in line 1259M, a phenomenon that has been

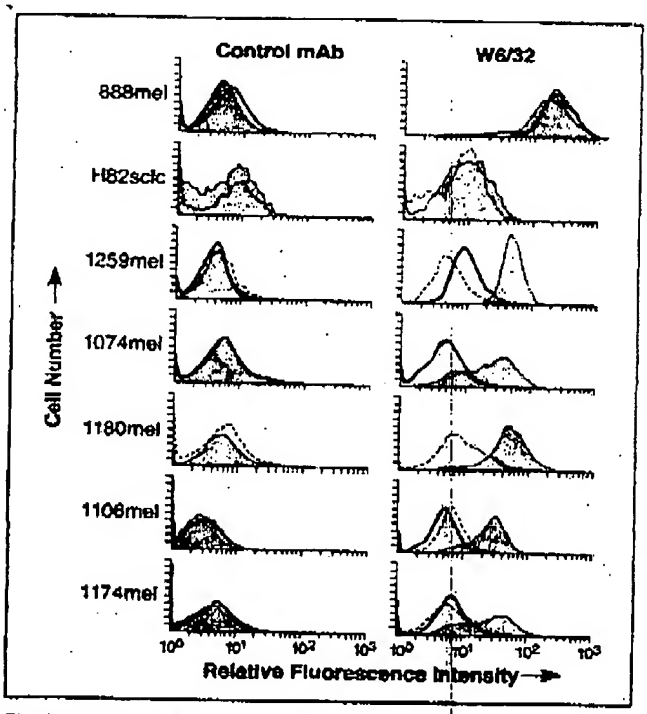

Fig. 1. Absence of major histocompatibility complex class I on the surfaces of metastatic human melanoma cell lines and restoration by transient expression of beta$_2$-microglobulin (β_2m). Fluorescence-activated cell sorter analysis was performed on tumor cell lines alone (heavy solid line), infected with wild-type vaccinia virus (dashed line), and infected with the recombinant vaccinia virus expressing β_2m (light solid line, shaded gray under the curve); cell lines were sequentially incubated with either the control immunoglobulin G2a antibody (left panels) or the W6/32 monoclonal antibody (right panels) and then with goat anti-mouse fluorescein isothiocyanate-conjugated Fab fragments. The cell lines analyzed were the positive control cell line 888mel (that is normal in its antigen-presenting capacity), the negative-control H82sclc (a small-cell lung cancer cell line that does not process antigens), and the five melanoma cell lines (1259mel, 1074mel, 1180mel, 1106mel, and 1174mel) that have lost functional β_2m. For two cell lines (H82sclc and 1180mel), only two curves are shown in this experiment because the tumor-cell-alone control was not performed because of inadequate numbers of cells.

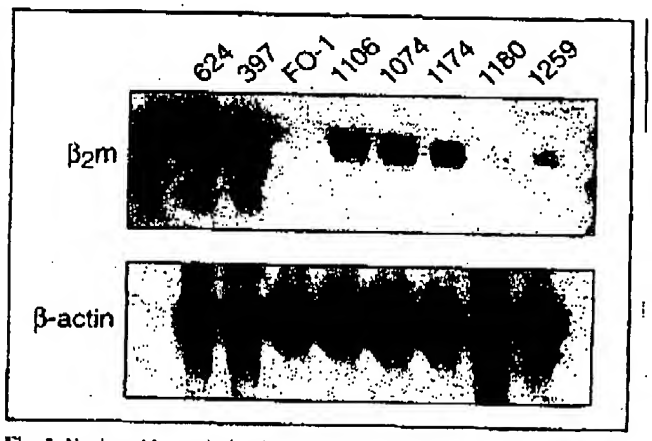

Fig. 2. Northern blot analysis of total RNA extracted from the indicated cells (624[mel], 397[mel], FO-1, 1106[mel], 1074[mel], 1174[mel], 1180[mel], 1259[mel]) and probed for the expression of beta$_2$-microglobulin (β_2m) messenger RNA. The northern blot probed for β_2m messenger RNA (top panel) was stripped and probed for β-actin to ensure sufficient loading of all lanes (bottom panel).

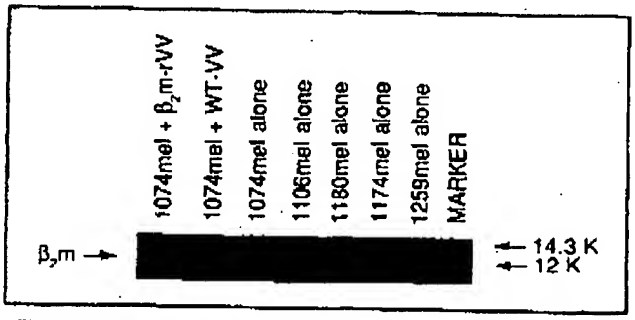

Fig. 3. Western blot analysis of total protein extracts for expression of immunoreactive beta$_2$-microglobulin (β_2m). Lysates of melanoma cell lines 1074mel, 1106mel, 1180mel, 1174mel, and 1259mel do not express any measurable immunoreactive β_2m. Line 1074mel is shown after infection with recombinant vaccinia virus expressing β_2m (left-most panel) or after infection with wild-type control vaccinia virus (second from left).

described by others (*45*). The melanoma cell line 888 was used as a positive control, since it exhibits normal antigen presentation; the small-cell lung cancer cell line H82 was used as a negative control, since it is defective in antigen processing, as previously reported (*14*).

The results of the western blot analysis, together with the cytofluorographic evidence, suggested that the genetic insertion of functional β_2m would fully restore the ability of the cell lines to process and present endogenous antigens for recognition by CD8+ T cells. On the other hand, if there were additional abnormalities in antigen processing, a full restoration of function in the antigen-presenting capabilities of the cell lines being studied would not be seen with the replacement of β_2m. To test this question directly, we employed a method for the study of antigen processing that was independent of both the HLA type of the tumor and the presence or absence of specific cellular proteins. With the use of a recombinant vaccinia virus encoding the mouse H-2 Kd MHC class I molecule (K^d-Vac), we tested human tumor cell lines for presentation of viral antigens to mouse K^d-restricted, vaccinia-specific $CD8^+$ T-cell populations and thus studied antigen-processing capabilities of human tumor cells per se. As shown in Fig. 4, restoration of the β_2m molecule in the tumor cells completely restored the antigen-processing capabilities of all five of the melanoma cell lines tested. The expression of β_2m had no effect on H82, the negative control in this experiment (*14*).

To test whether lack of functional β_2m was the sole mechanism of escape of these tumors from recognition by CD8+ T cells, we studied whether restoration of β_2m in these cell lines was associated with the restoration of killing of these cell lines by antitumor CD8+ T cells. This was a critical question because the loss of β_2m could be accompanied by the loss of tumor-associated antigens, making the restoration of β_2m in these cell lines a less relevant aspect of therapy. The problem with testing this question was that autologous TILs were not available from patients whose tumors gave rise to these cell lines. Since one of

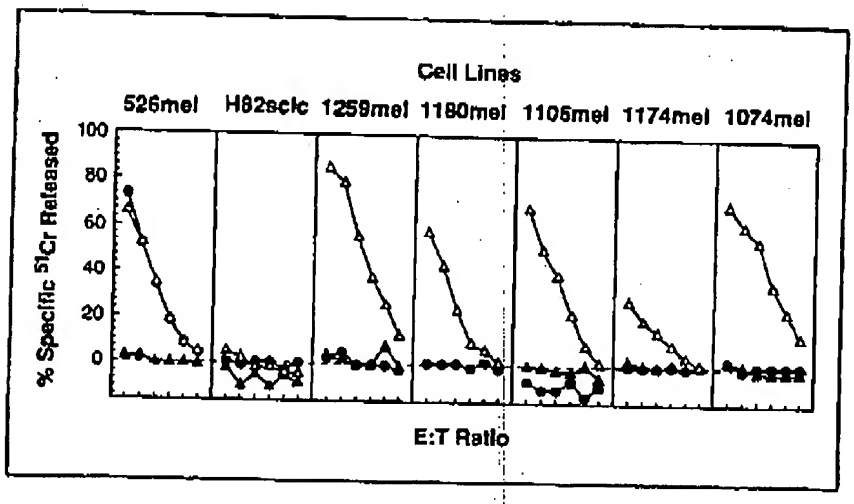

Fig. 4. Microcytotoxicity assay evaluating the antigen-presenting capacities of beta$_2$-microglobulin (β_2m)-deficient cell lines. Cell lines were screened for their capacities to present vaccinia virus antigens in the context of a transiently expressed H-2 K^d molecule. Effector cells (E) were vaccinia virus-specific, H-2 K^d-restricted murine cytotoxic T lymphocytes. Target cells (T) were either uninfected (▲), infected with vaccinia virus expressing H-2 K^d alone (●), or in combination with recombinant vaccinia virus expressing β_2m (△). The E:T ratios for the six points in each curve, reading from left to right, were 100:1, 50:1, 25:1, 12.5:1, 6.25:1, and 3.125:1. The melanoma cell line 526 (the positive control) is an antigen-processing intact cell line that processes vaccinia antigens efficiently in the absence of β_2m. The small-cell lung cancer cell line H82sclc fails to process antigens even in the presence of β_2m, since the antigen-processing lesions in this cell line are elsewhere (*14*). Note that 1259mel, 1180mel, 1106mel, 1174mel, and 1074mel all fail to present vaccinia virus antigens but can do so efficiently when functional β_2m is expressed endogenously.

the five patients lacking functional β_2m expression was HLA-A2.1 positive (No. 1074) and since we have generated CD8+ T cells that were capable of recognizing ubiquitously expressed melanoma tumor-associated antigens in the context of HLA-A2.1 (*46*), we could directly test whether restoration of β_2m led to immune recognition by CD8+ T cells. As shown in Fig. 5, expression of functional β_2m in the 1074mel cell line restored its susceptibility to lysis by HLA-A2.1-restricted antimelanoma CD8+ T cells.

Despite the lack of expression of cell-surface MHC class I molecules, all five of the β_2m-deficient melanoma cell lines were capable of being lysed by lymphokine-activated killer cells (data not shown).

To test the possibility that these observations were due simply to an artifact of the culture of the melanoma cells, we sought to assess the status of MHC class I expression of these tumors in vivo. Since a portion of each of the fresh melanoma specimens from which the melanoma cultures were developed was sent for pathologic analysis, we procured the paraffin-embedded tissue blocks and performed immunohistochemical analysis for the presence of β_2m. Tissue sections in all five of the cases in question were found to be uniformly devoid of β_2m (Fig. 6 and data not shown). Note that each tumor cell line contains the positive control of infiltrating cells that make up the vasculature of the tumor. Thus, the cells of the lines under scrutiny did not express β_2m in vivo that was capable of complexing with MHC class I α chains and stabilizing them on the tumor cell surfaces.

Finally, archival tumor sections obtained from patients prior to immunotherapy were available for three patients (Nos. 1106, 1180, and 1259) and were found to be β_2m positive (Table 1, Fig. 6, and data not shown). This result was consistent with the hypothesis that the loss of β_2m resulted from immunotherapy.

Discussion

The five cell lines described in this article were independently derived from metastatic melanomas obtained from five patients who had undergone immunotherapy. The tumors from which these five cell lines were derived may escape immune recognition by $CD8^+$ T cells via the loss of expression of functional β_2m, since replacement of β_2m fully restores the antigen-processing capabilities of all of these cell lines (Fig. 4) and can restore the ability of antitumor CD8+ T cells to recognize the cell line in the tumor (1074mel) that was matched for HLA-A2.1. Importantly, the loss of β_2m by these tumors was also observed in vivo by immunohistochemical analysis.

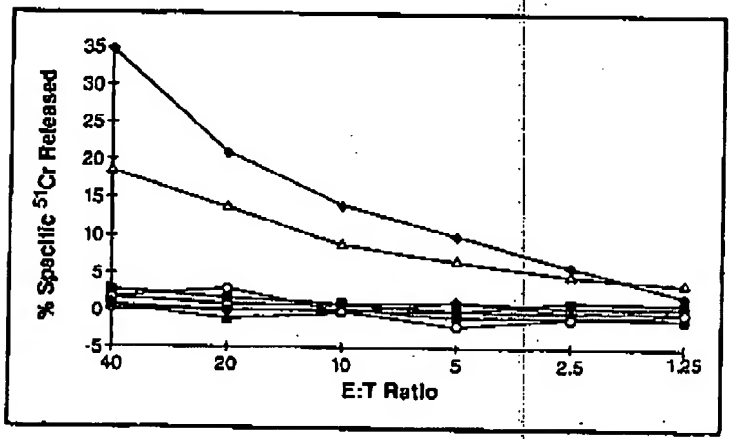

Fig. 5. Microcytotoxicity assay evaluating the capacity of a beta$_2$-microblobulin (β_2m)-deficient cell line to present tumor-associated antigen after restoration of β_2m. Melanoma cell lines were tested for recognition by HLA-A2.1-restricted antimelanoma effector (E) cells. Target cells (T) were the HLA-A2.1-positive, β_2m-positive 526 cell line (◆); the HLA-A2.1-positive but β_2m-negative 1074 cell line infected with control vaccinia virus (▲) or infected with recombinant vaccinia virus-expressing β_2m (△); the HLA-A2.1-negative but β_2m-positive 888 melanoma cell line with control (□) or β_2m vaccinia virus (■); and the HLA-A2.1-negative and β_2m- negative 1259 cell line with control (●) or β_2m vaccinia virus (○).

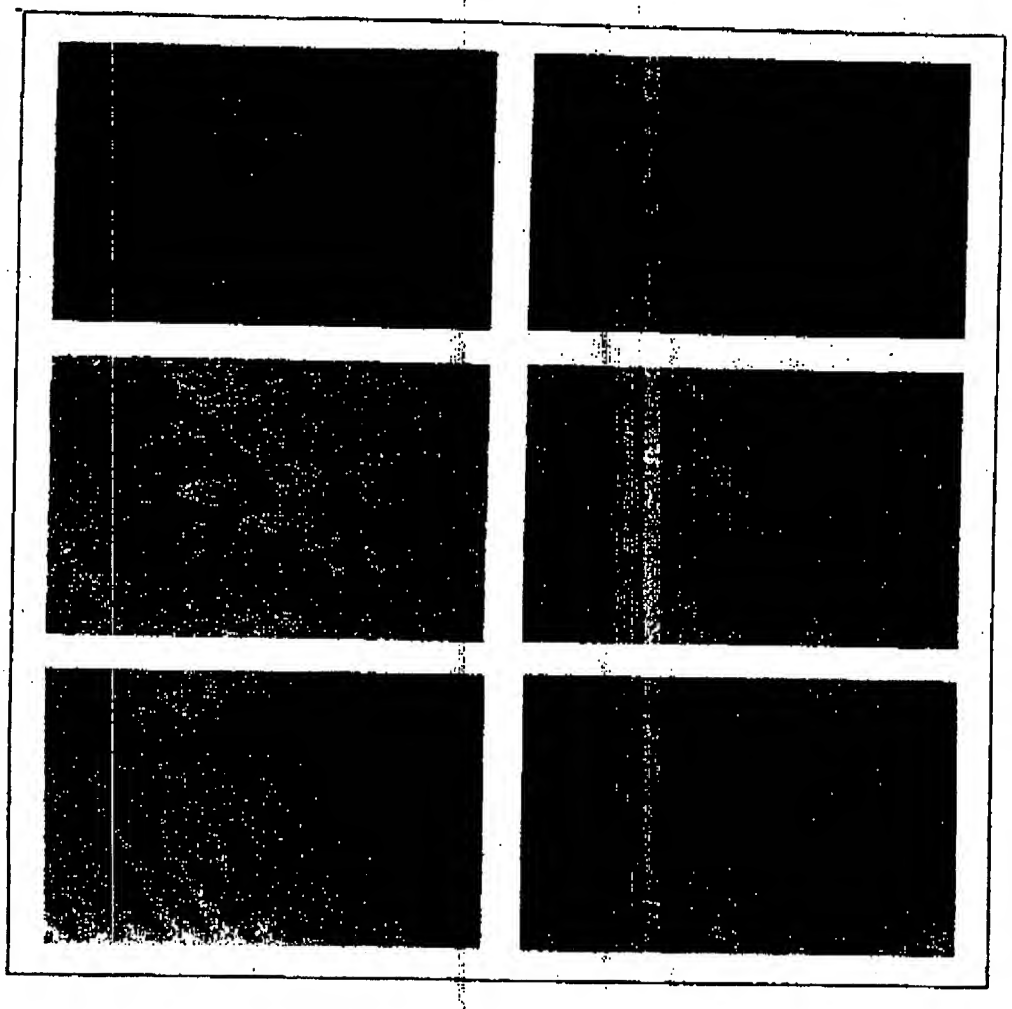

Fig. 6. Immunohistochemical staining of sections of melanoma in situ. Sections from tumor specimens described in Table 1 from patient Nos. 1106 (panels A-C) and 1180 (panels D-F) are shown. Immunohistochemical staining in panels B, C, E, and F was done at the same time, under exactly the same conditions with anti-human beta$_2$-microglobulin (β_2m) monoclonal antibody followed by immunoperoxidase staining (*see* "Materials and Methods" section for details). Strongly positive and weakly positive staining for β_2m is shown in panels B and E, respectively. Panels C and F are examples of specimens that stain negative for β_2m. Hematoxylin–eosin stains are shown in panels A and D, where melanin deposits that appear dark brown can be seen.

The loss of β_2m may be an important mechanism for the escape of some melanoma cells from recognition by CD8+ T cells and could explain why some patients experience a recrudescence of tumor growth, often in aggressive and ultimately lethal forms, after excellent responses to T-cell-based therapy. Indeed, none of the patients responded to immunotherapy subsequent to the observed loss of functional β_2m, and all of these five patients (Table 1) have died of disease that was noted to be clinically very aggressive. Perhaps most importantly, antitumor CD8+ T cells could not be grown from any of these lesions once there was loss of β_2m. However, the sample is too small to enable us to definitively draw the statistical conclusion that patients bearing tumors deficient in functional β_2m have poor outcomes compared with patients whose tumors do not suffer such a loss. Furthermore, we do not have enough illustrative examples to conclude that immunotherapy, or T-cell-based immunotherapy, was responsible for the loss of β_2m. Many patients whose tumors do express MHC class I fail to respond, and thus tumors are likely to use multiple different mechanisms to evade immune destruction. Nevertheless, the findings presented here could be used as the basis for prospective studies of much larger cohorts of patients receiving immunotherapy that could provide more definitive answers to these questions.

The incidence of the loss of functional β_2m in this study, four (31%) of 13, is strikingly high. However, loss of MHC class I expression in a cohort of patients undergoing immunotherapy with IL-2 has not yet been performed. Abnormalities in MHC class I expression in the absence of immunotherapy may be much higher than has been described in the earlier literature, as indicated by more recent studies examining particular allospecificities (*36,47*). Furthermore, the loss of β_2m has recently been associated with a mutator phenotype, frequently an early event in the development of carcinoma. The molecular basis for the loss of β_2m in these patients, particularly as it relates to microsatellite instability, is now being explored (*48*).

We have previously reported poor expression of MHC class I molecules by human small-cell lung cancers and an experimental mouse sarcoma (*14,38,49*). In our previous studies of deficient MHC class I expression, all of the small-cell lung cancer cell lines could be induced to express MHC class I by the addition of interferon gamma (IFN γ). We could not induce MHC class I expression with the use of IFN γ in the five human

melanoma cell lines reported here (data not shown). Thus, there appears to be histologic specificity in the mechanisms underlying a failure of recognition by CD8+ T cells: Six of six small-cell lung cancers previously studied presented antigen poorly because of a down-regulation of molecules important in antigen processing, while loss of functional β_2m was responsible for this effect in five of five melanomas in the present study.

The loss of β_2m is a highly effective mechanism of tumor escape from recognition by CD8+ T cells, since MHC class I molecules are not stable in its absence. The loss of expression of both copies of β_2m is also an efficient mechanism of escape, since loss of expression of any two other genes involved in antigen processing, such as TAP or proteasome components, would cause a much less profound loss of antigen-presenting capacity (*13,50*). Loss of β_2m may result in increased susceptibility to lysis by natural killer (NK) cells (*51-53*). However, the aggressive lethality of each of the five β_2m-deficient tumors reported in this study suggests either that lysis of these tumors by NK cells is ineffective or that the tumor cells have additional mechanisms of escape from immune recognition by NK cells.

Positive expression of β_2m mRNA by northern blot analysis suggested that the deletion of both copies of the gene was not responsible for the loss of functional β_2m and that mutation of one or both copies was probably responsible for the complete loss of functional β_2m. The absence of β_2m shown by western blot analysis indicated that the epitopes recognized by the MAb were not present to any measurable extent. Since western blot analysis measures the steady-state level of antigen, either the epitope was not produced because of mutation or premature termination or the protein produced had a vastly reduced half-life because of enhanced proteolysis due to a change in sequence or to a misfolding of the protein.

Studies in experimental murine models indicate that loss of β_2m expression by germline transmission of a homozygous disruption of the β_2m gene is not lethal (*54*). The β_2m-deficient mice express little or no functional class I antigen, and while other T-cell subsets were found in their normal distribution, the mice were found to have no mature CD8+ T cells (*29*). It is interesting that these mice were still capable of rejecting skin grafts (*55*) and were able to clear some viral infections (*56,57*). These findings are consistent with a view of the immune system as highly redundant. Indeed, all of the β_2m-loss mutants remained sensitive to lysis by lymphokine-activated killer cells, suggesting that immunotherapy based on non-MHC-restricted effector cells may be more effective in selected patients.

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Notes

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Tumor Escape from Immune Recognition

Lethal Recurrent Melanoma in a Patient Associated with Downregulation of the Peptide Transporter Protein TAP-1 and Loss of Expression of the Immunodominant MART-1/Melan-A Antigen

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Abstract

In the last few years, mutiple protein target antigens for immunorecognition by T cells have been identified on human melanoma. How melanoma lesions escape from functional antigen-specific immune recognition remains poorly understood. We have identified the concomitant loss of the immunodominant T cell–defined MART-1/Melan-A antigen and downregulation of the TAP-1 gene in a recurrent metastatic melanoma that was resected in 1993. This phenotype was not observed for an earlier autologous melanoma lesion resected in 1987. The "antigen loss" could be restored in the variant tumor cell line by simultaneously providing both the MART-1/Melan-A gene (by retroviral transfer) and the TAP-1 gene (by a bioballistic approach) resulting in tumor cell sensitivity to MART-1/Melan-A–specific cytotoxic T lymphocytes. This suggests that tumor escape from immune surveillance may have occured in vivo as a sequential result of (*a*) antigen loss, and (*b*) downregulation of the peptide-transporter protein TAP-1 expression by this patient's tumor over a 6-yr period from 1987 to 1993. These results suggest that the characterization of the T cell response to melanoma in individual patients and definition of the immunologically relevant genetic defects in tumors may be required to select the most effective therapeutic strategies for a given patient. (*J. Clin. Invest.* 1996. 98:1633–1641.) Key words: tumor antigens • T cell recognition • TAP • human melanoma • antigen processing

Introduction

Melanoma is perhaps the tumor for which the best evidence exists for an effective immune response, with many tumor-bearing patients living for prolonged periods before succumbing to metastatic disease. It is also currently the tumor that has been most successfully treated with biologic response modifiers such as IL-2 and with the adoptive transfer of ex vivo–expanded patient T cells specific for tumors (1).

It is now generally accepted that T cells are the major immune effectors regulating tumor progression in an immunocompetent host. T cells derived from patients with cancers have been particularly useful in defining the molecular targets of immune reactivity, such as the melanoma-associated antigens MART-1/Melan-A (2–5), gp100/Pmel17 (6–8), or tyrosinase (9, 10). Recent advances in our understanding of tumor immunobiology have allowed for the identification of cellular mechanisms by which tumors may escape immune recognition (Table I) despite the presence of tumor-reactive T-lymphocytes in patients.

We describe a female patient diagnosed in 1976 with primary melanoma treated with multiple sequential surgical excisions. Tumor cells were available from resected lesions in 1987 and 1993. Cultured autologous HLA–A2+-restricted tumor-infiltrating lymphocytes (TIL)[1] were derived from the 1993 abdominal lesions. These TIL recognized not only the autologous tumor samples that had been resected in 1987 and 1993, but also other HLA–A2+-matched allogeneic melanomas. While tumor cells obtained from the 1987 lesion were recognized by a panel of allogeneic HLA–A2-restricted clonal and polyclonal cytotoxic T-lymphocytes (CTL) lines specific for recently identified CTL-defined antigens shared among melanoma cells and melanocytes (MART-1/Melan-A and gp100/Pmel17), the tumor cells resected in 1993 were not. These observations support the critical importance of T cell selective pressure against tumor progression and document two concurrent mechanisms of tumor evasion of immunity in vivo.

Methods

Cellular reagents. We describe in this report the characteristics of two melanoma lesions obained from a female patient diagnosed in 1976 with primary melanoma. She underwent primarily surgical resection of the melanoma lesion and subsequently presented with localized metastases. She was treated with various experimental therapies including monoclonal antibodies to p97 (11) and high dose IL-2. After a treatment response to IL-2, the patient subsequently progressed with isolated cutaneous and nodal metastases until 1993 when she relapsed, presenting with small bowel metastases, and ultimately succumbed to the disease. The melanoma cells examined included a freshly harvested single cell suspension (SCS), designated 717 from a lesion resected in 1987, an SCS (6129TW) obtained from resected small bowel lesions, and the derived melanoma cell line designated PM1-B1. Single cell suspensions from melanoma lesions and TIL were prepared as described in detail previously (12) and were stored

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1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; FISH, fluorescence in situ hybridization; RT, reverse transcription; SCS, single cell suspension; TIL, tumor infiltrating lymphocyte.

Table I. Potential Defects in Melanoma Leading to Escape from Immune Recognition

Defect	Effects
Antigen loss or epitope loss in melanoma	Specific target of T cell recognition lost (29–31)
Point mutation within the MHC-presented T cell epitope	(I) Mutation abrogates binding to MHC molecules: target of T cell recognition lost (36). (II) Mutation does not affect binding to MHC: target of T cell recognition may be lost, or may function as 'partial agonist', or 'antagonist' leading to T cell anergy or to a qualitatively different effector T cell function(s) (33, 34).
Beta-2 microglobulin loss	Global defect in melanoma class I expression and CD8+ T cell recognition. MHC class II expression is intact (40, 43).
MHC class I global or allelic loss of MHC molecules	Global, individual decreased, or absent MHC class I expression on melanoma (such as HLA-A2, or B-locus alleles). Restricting MHC molecule for melanoma peptide presentation might be lost (40, 44, 45).
Peptide transporter defect	Decreased or absent expression of TAP-1. Failure in transporting peptides into the endoplasmic reticulum and expression of endogenously processed and presented MHC/peptide complexes on the cell surface. HLA-A2 expression is partially maintained due to binding of peptides derived from protein signal sequences in the ER (18, 19, 39, 40).
Downregulation of adhesion molecules, or costimulatory molecules	Delivery of signal 1 (MHC + peptide), but not signal 2 (costimulatory molecule) may induce T cell anergy. Loss of adhesion molecules may abrogate conjugate formation and consequently effector T cell functions (46).
Suppression of endothelial vascular adhesion molecule I (VCAM-1I) by melanoma	Downregulation of VCAM-1 prevents invasion of immune effector T cells to enter the tumor site (47).

in liquid nitrogen. Autologous TIL were maintained in AIM-V medium (GIBCO BRL, Grand Island, NY) supplemented with 1,000 IU IL-2 (provided by Cetus Corp., Emeryville, CA) in vitro without restimulation with tumor. Different effector CTL included the allogeneic polyclonal TIL line 1235, which has been previously used to identify the immunodominant HLA-A2-presented MART-1/Melan-A antigen (2–5), kindly provided by Dr. J.R.Yanelli (Surgery Branch, National Cancer Institute, Bethesda, MD). The HLA-A2-restricted CTL clones 1.1 and A83 have been described previously and exhibit similar specificity to that of TIL 1235, recognizing three closely related peptides provided by MART-1/MELAN-A (2, 5, 13). The CTL clone 2.37.1 recognizes a different set of melanoma peptides presented by HLA-A2 that are presumably derived from the gp100-Pmel17 protein. CTL targets included HLA-A2-matched melanoma cell lines and the LAK/NK sensitive target cell lines Daudi and K562 (obtained from Dr. Theresa Whiteside, IMD Laboratory at Pittsburgh Cancer Institute, Pittsburgh, PA). Recipient target cells in peptide pulsing assays included the human HLA-A2+ T/B cell hybrid cell line T2.

Cytogenetic analyses by fluorescence in situ hybridization (FISH). Metaphase cells were obtained from a melanoma cell line established from the melanoma single cell suspension resected in 1993 (called PM1-B1) using classical cytogenetic techniques (14). The chromosomes were trypsin-Giemsa banded. 20 metaphase cells were karyotyped and chromosomal abnormalities were expressed according to the International System for Cytogenetic Nomenclature (14). Cells labeled 717 (from the subcutaneous lesions resected in 1987), and 6129TW (from 1993) were melanoma cells, freshly dissociated after surgical excision and immediately cryopreserved in 10% DMSO in fetal bovine serum. The PM1-B1 line was initiated at the time of dissociation from a separate aliquot of the cell suspension that was labeled 6129TW and cryopreserved for later analysis. The cells were thawed, washed, and fixed in 3:1 methanol/acetic acid for 20 min at room temperature and slides were prepared. In preparation for FISH hybridization and analysis, the cell suspension from the PM1-B1 cell line (established from the 1993 lesion) and single cell suspensions 717 (1987), 6129TW (1993), and a control bone marrow specimen were applied to slides as described and treated according to the manufacturer's instructions (Oncor, Inc., Gaithersburg, MD). Pairs of satellite chromosome probes, one labeled with digoxigenin and the other with biotin for chromosomes 1 (D1Z5) and 3 (D3Z1), 1 and 7 (D7Z1), X (DXZ1), and 3, X, and 7, were prepared for hybridization. After hybridization, slides were washed and visualized using fluorescein-avidin/biotinylated antiavidin staining followed by rhodamine anti-digoxin antibody. The slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in antifade solution and 250 interphase nuclei per pair were analyzed and photographed using a BHS microscope (Olympus Corp., Lake Success, NY) equipped with epifluorescence optics and a triple bandpass filter (digoxigenin/fluorescein/DAPI). Color photographs were taken on Ektochrome 100-HC color film (Eastman Kodak Co., Rochester, NY).

T cell "epitope mapping" of melanoma cell lines. MHC class I bound peptides were removed from viable tumor cells by mild acid elution leading to dissociation of MHC class heavy chains, beta-2 microglobulin, and peptides. Peptide material was fractionated by HPLC as previously described in detail (15, 16) and aliquots of individual HPLC fractions (containing peptide material) were pulsed on HLA-A2+ nonmelanoma antigen-presenting cells (i.e., the human T/B cell hybrid T2). Individual HPLC fractions were tested for T cell recognition in a standard 4-h ^{51}Cr release assay as described in detail (15, 16).

Reverse transcription–PCR analysis. Total RNA was prepared from tumor cells according to Chomczynski and Sacchi (17), reverse transcribed into cDNA, and tested for integrity using β-actin–speci-

fied primers. The primer for detection of the melanoma antigen MART-1/Melan-A has been reported previously (3), and the primer panel for genes involved in peptide transport (TAP1/2) and peptide generation (LMP2/7) has been described in detail elsewhere (18).

Immunostaining. The polyclonal rabbit antibody specific for the human TAP-1, but not TAP-2 (19) molecule was a kind gift of Dr. Hidde Ploegh (Massachusetts Institute of Technology, Cambridge MA) and was used for staining cytospins at a 1:1,000 dilution in PBS. Briefly, single-cell suspensions were prepared from the PM1-B1 line, and a single-cell suspension resected from 1987 (717) or from 1993 (6129TW) was blocked with nonimmune serum (1:10 in PBS), incubated for 5 h at room temperature with the anti–TAP-1 Ab, washed, incubated for 30 min with an $F(ab)'_2$ goat anti–rabbit Fc fragment, incubated for 30 min with ABC complex (Vectastain; Vector Laboratories, Inc., Burlington, CA), washed, incubated 9 min with aminoethylcarbozol (AEC; Biomeda, Foster City, CA), counterstained with hematoxylin, covered, and dried at 80°C.

Gene transfer. The human TAP-1 gene, cloned in the RSV5 vector was a generous gift from Dr. Thomas Spies, (Massachusetts Institute of Technology) and has been reported to restore TAP-1 function in human cell lines (20). The TAP-1 gene was delivered into melanoma cells by a bioballistic approach using TAP-1 DNA–coated gold particles that are accelerated by high air pressure to penetrate target cells. The acceleration device "gene gun" (Accell®) was provided by Agracetus Inc. (Middletown, WI). DNA coating of gold beads and acceleration was performed according to the manufacturer's instructions. The HLA–A2 gene was provided by Dr. R. Salter (Department of Pathology, University of Pittsburgh) and the full length (399 bp) human MART-1/Melan-A gene was cloned by reverse transcription (RT)-PCR using cDNA derived from the MART-1/Melan-A positive cell line Mel 624 and subcloned into the MFG-based proviral vector containing the selectable marker for G418 (geneticin) resistance (termed DFG-MART-1/Melan-A). Infectious retroviral supernatant was prepared as described in detail elsewhere (21) and the DFG-MART-1/Melan-A–infected melanoma line PM1-B1 was selected for 3 wk in CMRL medium (GIBCO BRL), supplemented with 400 μg/ml G418 (Gibco Laboratories, Grand Island, NY).

Results

TIL–PM1-B1 (1993) recognizes a subdominant T cell epitope on the autologous tumor. TIL derived from patient PM1-B1 in 1993 were grown in vitro for 4 wk, without restimulation with autologous or allogeneic tumor cells. TIL exhibited HLA–A2-restricted lysis of tumor targets in a standard 4-h ^{51}Cr release assay (data not shown). For a more detailed analysis of TIL specificity, MHC class 1 bound peptides were acid eluted from the autologous tumor cell line PM1-B1 or the

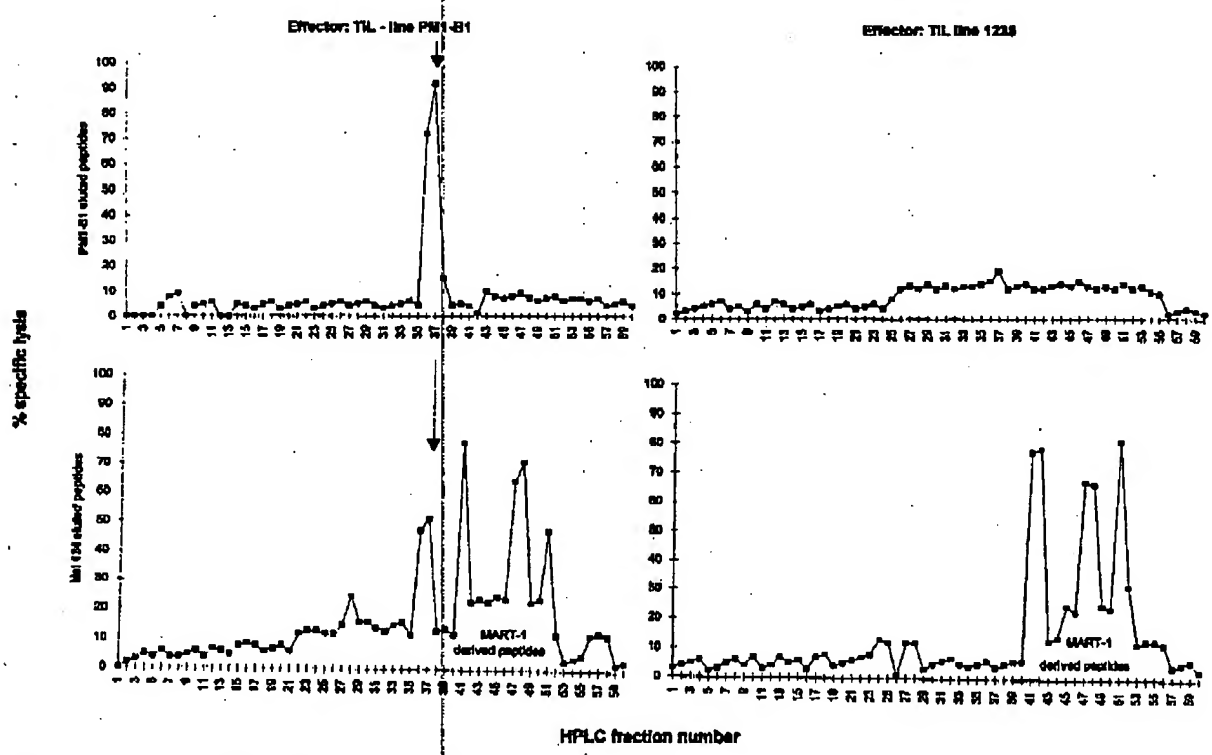

Figure 1. T cell epitope mapping: MART-1/Melan-A–derived T cell epitopes are absent in PM1-B1 (1993). Peptides were eluted from either the tumor cell line PM1-B1 or the HLA–A2+ control melanoma 624, and HPLC fractionated. Individual HPLC fractions were pulsed on the HLA–A2+-recipient target cell line T2 and tested for T cell recognition by cytotoxicity using either the autologous TIL line PM1-B1, or the MART-1/Melan-A–specific TIL line 1235 as effector T cells. TIL 1235 fails to detect MART-1/Melan-A–provided epitopes on PM1-B1, which are present on the control Mel line 624 eluting in HPLC fractions 41/42, 47/48, and 51. The TIL line PM1-B1 also recognizes such MART-1/Melan-A–derived bioactive HPLC fractions using peptides from the allogeneic Mel line 624. Such peptides are absent on the autologous tumor PM1-B1. Additionally, the TIL PM1-B1 recognizes a different HLA–A2-presented peptide(s) eluting in HPLC fraction 37 from the autologous tumor, and from the HLA–A2+ allogeneic Mel 624 (*arrow*).

HLA-A2+-matched allogeneic melanoma cell line 624, and HPLC fractionated. Aliquots from each individual HPLC fraction were pulsed onto HLA-A2 nonmelanoma targets (i.e., T2) and tested for T cell–mediated cytotoxicity using the autologous TIL line PM1-B1 or the MART-1/Melan-A–specific TIL line 1235 as effector cells (Fig. 1). TIL line PM1-B1 recognizes only a single dominant bioactive peptide peak eluting in HPLC fraction 37 (82% specific lysis) when peptides from the autologous tumor PM1-B1 were pulsed onto the HLA-A2+ target cell line T2. This target peptide had not been previously identified as a melanoma TIL epitope in our previous series of other HLA-A2+ melanomas, but a careful retrospective analysis of all available data suggests that this bioactive HPLC fraction contains a "subdominant" peptide epitope recognized by freshly harvested TIL (13, 16), but not by long-term cultured TIL. In marked contrast, TIL line PM1-B1 recognizes four bioactive HPLC peaks, containing Mel 624 cell-derived peptides, eluting in the corresponding HPLC fraction 37, but also three additional peaks eluting in fractions 41/42, 47/48, and 51. The MART-1/Melan-A–specific CTL line TIL 1235 did not recognize any bioactive peptide material eluted from Mel PM1-B1, but recognized three bioactive HPLC peaks eluting in HPLC fractions 41/42, 47/48, and 51, which contain T cell epitopes eluted from Mel 624 cells pulsed onto T2 cells. The bioactive HPLC peaks eluting in HPLC fractions 41/42, 47/48, and 51 appear to contain three closely related peptides

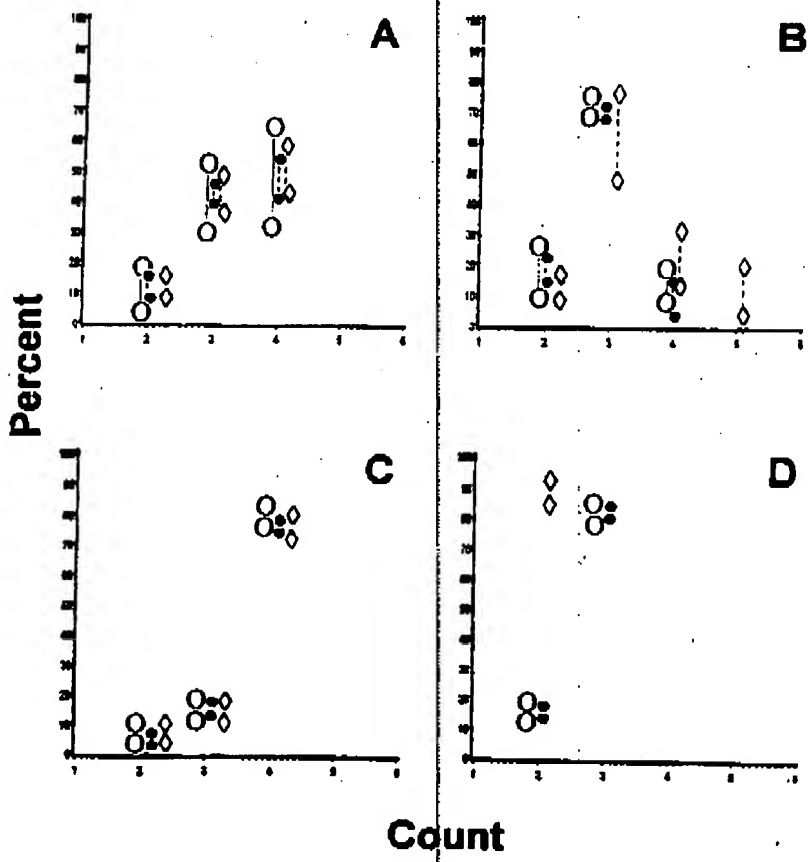

Figure 2. Similar cytogenetics of two different tumor cell preparations obtained in 1987 and 1993: graphic illustration of similarities in copy of chromosomes 1, 3, 7, and X in the two directly harvested cell suspensions, 6129TW (○), 717 (●), and the cell line PM-B1 (◇). The tumor cell line PM1-B1 was established from an SCS resected in 1993 (6129TW). We could not establish a melanoma cell line from the melanoma specimen obtained in 1987, hence only the SCS (717, 1987) was available for subsequent immunologic testing. To evaluate (*a*) similarities or gross changes in chromosome numbers between the tumor specimens obtained between 1987 and 1993, and (*b*) if the melanoma cell line PM1-B1 is representative of the SCS 6129TW (1993), we performed FISH analysis for the copy number of chromosomes 1, 3, 7, and X in the cultured PM1-B1 cell line, and the freshly dissociated and cryopreserved (> 97% tumor cells) SCS 717 and 6129TW. The graphs show the results of two independent determinations of chromosome copy number frequency. Pairs of satellite chromosome probes, one labeled with digoxigenin and the other with biotin (pairs 1+3, 1+7, X+3, and X+7) were hybridized to slides and counterstained with 4,6-diamid-ino-2-phenylindole as described in Methods. 250 interphase nuclei per pair were analyzed and photographed. The individual chromosome counts are presented as follows: (*A*) chromosome 1; (*B*) chromosome 3; (*C*) chromosome 7; and (*D*) chromosome X. The counts for individual satellite chromosome probes showed constant results in two independent experiments. For instance, the symbols for the individual cell populations examined (717 SCS [1987], *closed circles*, 6129TW SCS [1993], *open circles*, and the 1993 established cell line PM1-B1, (*diamonds*) are very close together in *C* and *D*, exhibiting almost identical counts in two independent experiments. However, we observed limited variation (*A* and *B*) for the chromomsome 1– and 3–specific probes indicated by bars or dotted lines. For instance in one experiment, examination of 250 interphase nuclei yielded 60% of cells showing four copy numbers of chromosome 1 for the cell line PM1-B1 (*A*, *diamonds*), and yet in the repeat, 44% of cells staining for chromosome 1. These data indicated that (*a*) three to four subpopulations are present within the freshly harvested tumor cell lines and within the cultured cell line PM1-B1 as defined by chromosome copy numbers of chromosomes 1, 3, 7, and X; (*b*) the freshly harvested SCS obtained from resected lesions in 1987 and 1993 appear to be closely related as regards the copy numbers of chromosomes 1, 3, 7, and X; and (*c*) the melanoma cell line PM1-B1 resembles (as regards the chromosome markers examined) the freshly harvested SCS obtained from the resected 1993 lesion. In general, the FISH data strongly suggested that copy numbers of chromosomes 1, 3, and 7 remained constant between all three cell populations examined. Note that in *D*, only the cell line PM1-B1 (*diamonds*) exhibits 90–94% of cells with two X chromosomes. In contrast, the freshly harvested tumor SCS from 1987 and 1993 (*open* and *closed circles*) exhibit 80–88% of cells with three X chromosomes, indicating alteration of the cell line PM1-B1 during in vitro cell culture.

Table II. T cell Epitopes Derived from MART-1/Melan-A Are Absent on the Tumor Resected in 1993, but Are Present on the Tumor Resected in 1987

		Mel-line 614 (control)		Mel line PM1-B1 (1993)		SCS 6129 (1993)		SCS 717 (1987)	
Effector TIL	Target antigen	Control IgG	W6/32	Control IgG	W6/32	Control IgG	W6/32	Control IgG	W6/32
E:T = 10:1		*Percent specific lysis*							
PM1-B1		50	0	20	0	13	0	50	0
TIL 1235	MART-1/Melan-A	37	1	2	1	0	0	28	2
TIL 1.1	MART-1/Melan-A	31	0	1	0	0	0	38	0
TIL A83	MART-1 Melan-A	27	2	0	0	0	0	20	0
TIL 2.371	gp100	18	1	1	0	0	0	16	0

To evaluate MHC class I–presented T cell epitopes on the control tumor Mel 624, the single cell suspension harvested in 1987 (SCS 17), the SCS harvested in 1993 (SCS 6129TW), and its derivative, the tumor cell line PM1-B1, were assayed for T cell recognition in a standard 4-h ^{51}Cr release assay. Specific T cell recognition was tested by blocking with an antibody directed against MHC class I (W6/32), or with control IgG. Four different CTL lines failed to kill the melanoma resected in 1993, but killed the tumor resected in 1987. Only the autologous TIL line PM1-B1 recognizes the tumor resected in 1993 (20% lysis).

provided by the MART-1/Melan-A antigen (2–5), which is expressed in melanoma cells and in normal melanocytes (2–5, 13, 22). Based on these T cell epitope–mapping experiments, it is suggested that the TIL present in the melanoma lesion resected in 1993 (PM1-B1) recognize MART-1/Melan-A–provided peptides, which are also recognized by the MART-1/Melan-A–specific allogeneic TIL line 1235. TIL PM1-B1 additionally recognizes an HLA–A2-presented melanoma T cell epitope eluting in HPLC fraction 37 that is expressed by both autologous and on HLA–A2-matched allogeneic melanoma cells. The sequence identity of its protein precursor is currently the focus of intensive study.

T cell epitopes defined by CTL clones are present on PM1-B1 (1987) but have been lost from PM1-B1 (1993). To compare the expression of T cell epitopes on the melanoma lesion resected in 1987 with the melanoma lesion resected in 1993, we tested several anti–MART-1/Melan-A or anti–gp100/Pmel17 CTL clones against a control target (Mel 624 cells), the freshly harvested SCS 717 (1987), SCS 6129TW (1993), and the melanoma cell line PM1-B1 (1993). Classical cytogenetic and FISH analysis for chromosomes X, 1, 3, and 7 revealed that the melanoma specimens resected in 1987 and 1993 are very closely related and that the melanoma cell line PM1-B1 is representative of the freshly isolated tumor cells harvested in 1993 (Fig. 2). TIL clones 1.1 and A83 are HLA–A2-restricted CD8+ CTL clones recognizing three closely related peptides provided by the melanoma antigen MART-1/Melan-A (2–5, 13, 22). The TIL clone 2.37.1 recognizes a different set of HLA–A2-presented melanoma peptides, eluting in HPLC fractions 27 and 33, presumably provided by the melanoma antigen gp100/Pmel17. Additionally, TIL PM1-B1 was evaluated for its ability to react against a panel of tumor target cell lines. All four TIL lines exhibited MHC class I–restricted lysis of the HLA–A2 control melanoma 624, and the SCS 717 resected in 1987 (Table II). In contrast, only the (autologous) TIL line PM1-B1 lysed the SCS 6129TW (1993) and its derived tumor cell line PM1-B1 (1993). MART-1/Melan-A–specific TIL clones A83 and 1.1, and the gp100-reactive TIL clone 2.37.1,

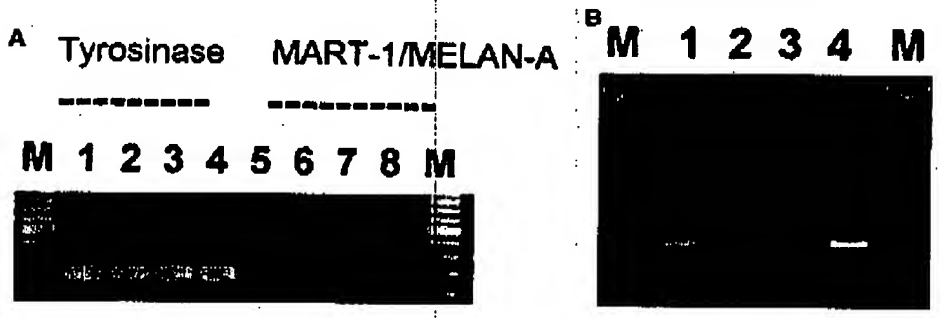

Figure 3. MART-1/Melan-A mRNA is absent in the melanoma resected in 1993 but present in 1987. (*A*) RNA was extracted, reverse transcribed into cDNA and tested for expression of tyrosinase, or MART-1/Melan-A by RT-PCR. (Lanes *1* and *5*) SCS 717 (1987), (Lanes *2* and *6*) SCS 6129TW (1993), (Lanes *3* and *7*) the derived Mel line PM1-B1 (1993), (Lanes *4* and *8*) control Mel 624. Primers employed to detect MART-1/Melan-A covered the entire gene product (399 bp). In summary, tyrosinase mRNA could be detected in the freshly harvested tumor cells obtained in 1987 (lane *1*) and 1993 (lane *2*), in the 1993 established cell line PM1-B1 (lane *3*), as well as in the positive control melanoma cell line 624 (lane *4*). In contrast, MART-1/Melan-A mRNA could be detected in the freshly harvested tumor cells from 1987 (lane *5*) and in the positive control melanoma cell line 624 (lane *8*). Freshly harvested tumor cells from the 1993 resected melanoma lesion (lane *6*) and the derived melanoma cell line PM1-B1 (lane *7*) tested negative for MART-1/Melan-A mRNA expression. A similar result was obtained using MART-1/Melan-A–specific primers spanning exons 2 and 5 (*B*). Freshly harvested tumor cells from 1987 (lane *1*) and the control melanoma 624 (lane *4*) showed MART-1/Melan-A expression. In contrast, freshly harvested tumor cells from 1993 (lane *2*), or the derived melanoma cell lines (lane *3*) showed low, or absent, MART-1/Melan-A mRNA expression.

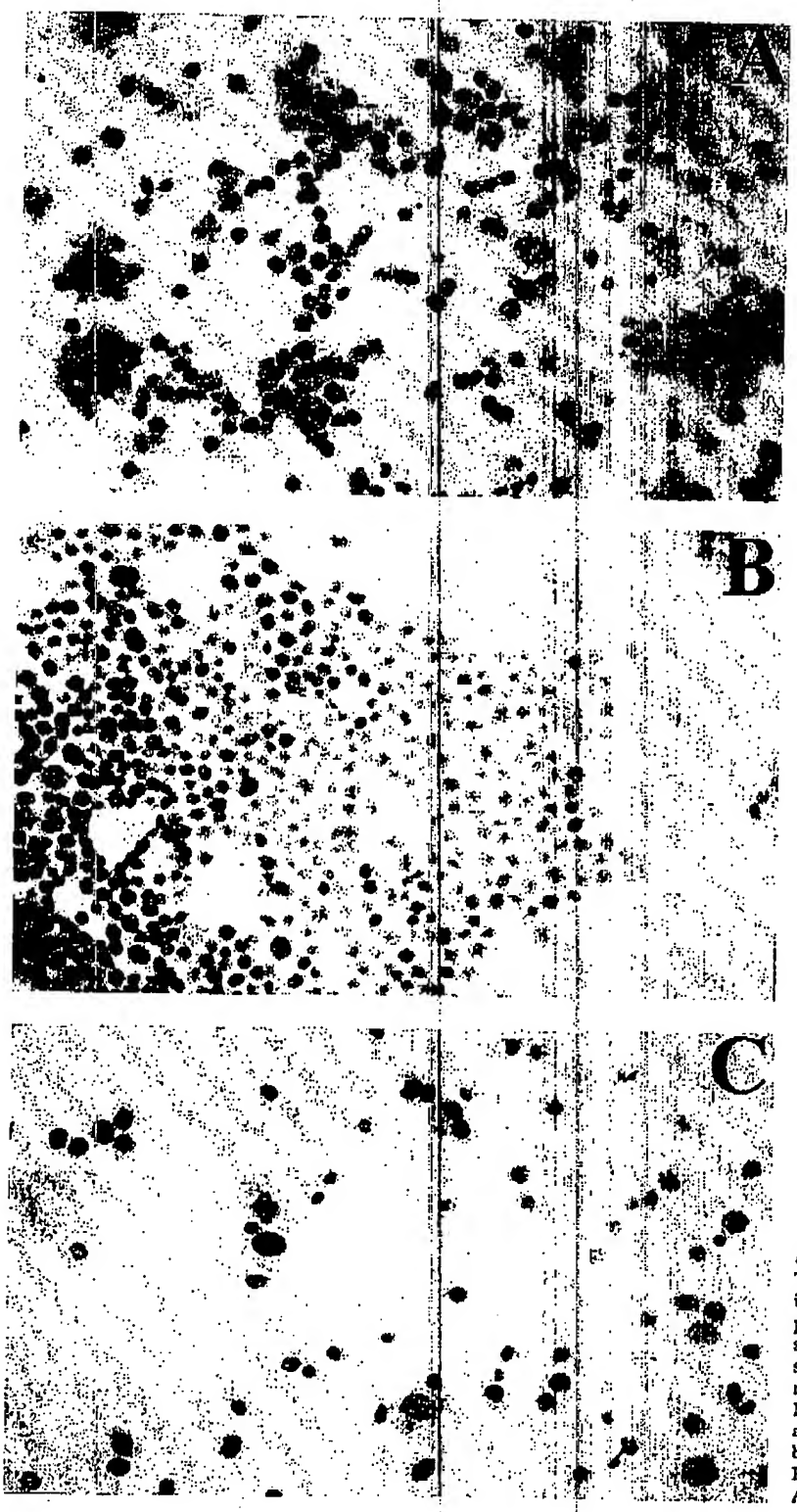

Figure 4. The peptide transporter molecule TAP-1 is expressed in the melanoma resected in 1987, but not in 1993. Cytospins were prepared and stained for TAP-1 protein expression (showing red staining) using an antibody specific for the TAP-1 protein. Melanoma cells resected in 1987 stained postive for TAP-1 (*A*). In contrast, TAP-1 protein is absent in the melanoma resected in 1993 (*B*), but could partially be restored (*C*) using the expression plasmid RSV5-TAP1 and the gene-delivery device Accell®.

failed to recognize 6129TW, or the melanoma line PM1-B1 (1993), confirming the absence of MART-1/Melan-A–derived T cell–defined epitopes presented on the tumor resected in 1993, but not in 1987.

Detection of antigen loss: MART-1/Melan-A mRNA is expressed by PM1-B1 (1987), but not by PM1-B1 (1993). RNA was extracted from the melanoma cells and reverse transcribed into cDNA to test for the presence of the MART-1/Melan-A mRNA. As a control, the message for tyrosinase, a key enzyme of melanin formation, was also evaluated (Fig. 3, *A* and *B*). All samples (717, SCS 1987; 6129TW, SCS 1993; Mel line, PM1-B1 1993; and the control, melanoma 624) exhibited a bright signal for tyrosinase. In contrast, only sample 717 (SCS, 1987) and the control melanoma 624 showed a positive signal for MART-1/Melan-A. Neither the freshly harvested SCS (1993) (6129TW) nor its derived cell line PM1-B1 (1993) expressed mRNA for MART-1/Melan-A.

Gene transfer of MART-1/Melan-A and TAP-1 into PM1-B1 (1993) restores the expression of MART-1/Melan-A epitopes recognized by CD8+ cytotoxic T cells. The MART-1/Melan-A gene was cloned from cDNA derived from (control) melanoma cell line 624. A retroviral vector with a selectable marker (Geneticin) termed DFG-MART-1/Melan-A was constructed for retroviral gene transfer of the MART-1/Melan-A gene. Examination of the antigen processing (protcasome subunits LMP2/LMP7) and presentation machinery (the peptide transporter heterodimer TAP1/TAP2) by RT-PCR analysis has revealed that TAP1 mRNA expression appeared to be downregulated in the melanoma lesion resected in 1993 compared with the tumor lesion in 1987. We could not sufficiently restore TAP1 mRNA by IFNγ (1,000 IU/ml, 3 d) treatment (data not shown). Therefore, the human TAP1 gene was delivered into melanoma cells by "shooting" the gene into the PM1-B1 cell line using the "gene gun" device Accell®. TAP1 gene expression could be monitored using a polyclonal Ab specific for the TAP-1 protein (Fig. 4). Staining of the melanoma sample 717 (1987) shows a high percentage of TAP1-positive staining tumor cells (Fig. 4 *A*). In contrast, the melanoma cell line PM1-B1 (1993) is deficient in TAP1 protein expression (Fig. 4 *B*). The single cell suspension 6129TW was similarly TAP-1 negative (data not shown). The expression of TAP-1 could be restored in ~ 20% of the PM1-B1 cells after gene gun delivery of the TAP-1 gene and selection for 10 d in G418 (Fig. 4 *C*). T cell recognition of PM1-B1 cells after TAP-1 gene transfer was examined using the polyclonal TIL line 1235 or the CTL clone A83, both specific for HLA–A2-presented peptides derived from MART-1/Melan-A. Both T cell lines recognized the positive control melanoma cell 624, but not the PM1-B1 (1993) cell line (Table III). Gene transfer of MART-1/Melan-A alone, or TAP-1 alone did not restore T cell recognition. In contrast, DFG-MART-1/Melan-A retrovirally infected PM1-B1 melanoma cells, into which the TAP-1 gene was additionally delivered by the bioballistic approach, were lysed by MART-1/Melan-A–specific TIL. The PM1-B1 cell line infected with a retroviral vector containing the HLA–A2 gene (DFG-HLA-A2) was not recognized by either effector CTL line. Based on these results, we concluded that in a melanoma resected from a patient in 1993 (compared to a lesion resected in 1987) there was (*a*) a loss of the immunodominant antigen recognized by the majority of HLA–A2-restricted and melanoma specific CD8+ CTL (MART-1/Melan-A), and (*b*) a lack of cytosolic peptide transport into the endoplasmic reticulum (ER), which is physiologically required for the assembly and transport of MHC class I molecules, as a result of impaired TAP-1 expression.

Discussion

Human melanoma cells typically express a number of "shared" antigens yielding peptide epitopes recognized by HLA–A2-restricted CD8+ cytotoxic T-lymphocytes. Such antigens are also shared among melanoma cells and their normal counterparts, melanocytes (2–10, 23–27). Each of these gene products are encoded by normal, nonmutated genes, with most of them providing several T cell epitopes, e.g., MART-1/Melan-A (three epitopes), gp100 (two epitopes), and tyrosinase (two

Table III. Simultaneous Expression of the MART-1/Melan-A Antigen with TAP-1 Restores CTL Recognition of Tumor PM1-B1 by HLA–A2-restricted and MART-1/Melan-A–specific CTL Lines TIL 1235 or CTL Clone A83

Targets	PM1-B1	PM1-B1	PM1-B1	PM1-B1	PM1-B1	Mel 624 (control)	SCS 6129 (1993)	SCS 717 (1987)
Gene transfer	—	DFG-HLA-A2	RSV-TAP1	DFG-MART-1	DFG-MART-1 + RSV-TAP-1			
Expression of								
TAP1	–	–	+	–	+	+	–	+
MART-1	–	–	–	+	+	+	–	+
Effectors:		E:T = 10:1			Percent specific Lysis			
TIL1235	0	0	1	8	27	36	0	30
TIL A83	0	0	1	6	17	27	0	20

The Mart-1/Melan-A antigen was expressed in Mel PM1-B1 cells using a retroviral vector termed DFG-MART-1/neo. TAP-1 expression was achieved using the expression plasmid RSV5-TAP1 and the gene-delivery device Accell®. After selection in G418, target cell lines were tested for CTL recognition by the HLA–A2-restricted and MART-1/Melan-A–specific TIL line 1235, or the CTL clone A83. A retroviral vector with the HLA–A2 gene (DFG-HLA-A2) served as a control. Expression of MART-1 alone showed 8 and 6% specific lysis, but simultaneous expression of both MART-1/Melan-A and TAP-1 resulted in significant CTL recognition. The HLA–A2+, MART-1/Melan-A+, Tap1+ melanoma cell line 624, as well as the SCS (freshly harvested single cell suspension) harvested from patient PM1-B1 in 1987, and in 1993 served as controls. Expression of TAP-1 in PM1-B1 did not result in significantly enhanced lysis of the autologous tumor (1993) by autologous TIL (data not shown). This observation may be due to the high frequency of MART-1/Melan-A–reactive CTL present within the autologous TIL. Such T cell effectors would not be able to recognize the MART-1/Melan-A negative, TAP-1+ PM1-B1 melanoma cell line.

epitopes) recognized by cytotoxic T cells. Recent studies revealed that 'private' tumor antigens recognized by cytotoxic T cells may result from mutated gene products; e.g., mutated CDK4 involved in cell cycle regulation (28). Such T cell–defined antigens provide excellent candidates for the development of novel tumor vaccines capable of eliciting antitumor immune responses in melanoma patients (6, 10) either in the form of native proteins or as peptide-based vaccines. Clinical trials implementing peptide-based vaccines have recently begun in the United States and in Europe.

Tumor antigen heterogeneity, expressed as quantitative differences in CTL recognition has been previously described in human melanoma, but only from a single tumor lesion and only after cloning the tumor cells in vitro (29, 30). We have described the loss of expression of a defined immunodominant antigen by a melanoma lesion in situ. Similar observations of antigen losses have been reported in murine models (31, 32) where elimination or downregulation of entire genes encoding tumor antigens was correlated with "antigen-loss" variants exhibiting decreased immunogenicity and increased tumorigenicity, i.e., in the mouse mastocytoma P815 model described by De Plaen et al. (31) and Van den Eynde et al. (32). However, in addition to the downregulation in expression of an entire tumor antigen providing peptides presented by MHC, more subtle changes may also occur. "Epitope-loss" variants induced by point mutations within a MHC class I–presented tumor antigenic peptide recognized by T cells may abrogate CTL recognition specific for the wild-type peptide (33). An additional mechanism for immune escape are point mutations within immunogenic individual peptides, which are still capable of binding to the proper MHC molecule, and still engage with the appropriate T cell receptor, but are capable of altering the quality and magnitude of the triggered T cell response (34, 35). Alternatively, point mutations within a given peptide may simply abrogate the capability of that peptide to bind to its proper MHC restriction element and hence preclude CTL reactivity (36, 37).

A striking observation in these studies is that we could demonstrate anti–MART-1/Melan-A–specific TIL from a melanoma cell, which apparently lost expression of the MART-1/Melan-A antigen. It is tempting to speculate that a vigorous antitumor CTL immune response in vivo might facilitate the generation of antigen-loss variants by eradicating tumor target cells expressing the MART-1/Melan-A antigen. Alternatively, the presence of anti–MART-1/Melan-A–directed TIL might also reflect the high immunogenicity of this particular antigen, since the T cell precursor frequency in PBL in HLA–A2+ melanoma patients, and even in normal healthy volunteers appears to be surprisingly high (38). Of note, the PM1-B1 TIL line recognized a single residual epitope (eluting in HPLC fraction 37) presented by the autologous tumor in 1987 and 1993 (Fig. 1 and Table II). This HLA–A2-associated T cell epitope appears to be shared by other HLA–A2+ melanoma cells (see Fig. 1).

Two observations appear to be of critical importance: (*a*) the immune system is capable of reacting against a "subdominant" epitope(s) (i.e., eluting in HPLC fraction 37 for PM1-B1, 1993) in the absence of the presumed immunodominant antigen presented by HLA–A2 (i.e., MART-1/Melan-A); and (*b*) this particular T cell epitope is presented in the absence of TAP-1; i.e., presumably independent of the requirement of peptide transport from the cytosol into the ER. This phenomenon appears to reflect a common feature of the HLA–A2 molecule, which has been reported to bind to leader-peptide sequences from various proteins, cleaved by proteases in the ER. Such leader-peptide sequences are independent of the active TAP-1 ATP-dependent peptide transport and represent a physiological mechanism of protein processing and "trimming" in the ER. One of the low affinity HLA–A2 binding peptides recognized by anti-melanoma–directed CTL has been reported to represent such a leader peptide cleaved during the physiologic processing of the enzyme tyrosinase in the ER (39).

Recent studies from Ferrone and Marincola (40) suggest that downregulation of TAP-1 expression is relatively frequent, at least in a proportion of cells (25–50%) within melanoma lesions. Alternatively, viral infection of human cells may lead to exclusive downregulation of TAP-1 (41, 42). TAP-independent peptide vaccines may be relevant in alternate tumor histologies where the peptide transporter molecules appear to be frequently downregulated, such as in lung (18) or cervical (19) cancer and underscore the need for individual examination of a patient's tumor lesions before immunotherapy.

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Functional Dissociation Between Local and Systemic Immune Response During Anti-Melanoma Peptide Vaccination

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Peptide vaccination against tumor Ags can induce powerful systemic CTL responses. However, in the majority of patients, no tumor regression is noted. To study this discrepancy, we analyzed CTL reactivity in a melanoma patient (F001) vaccinated with g209-2M peptide, a single residue variant of $gp100_{209-217}$. G209/g209-2M-reactive CTL were identified in post- but not pre-vaccination PBL. Limiting dilution analysis identified one predominant CTL clone (C1-35), with TCR Vβ6s2, recognizing g209/HLA-A*0201-expressing targets. Additionally, two autologous melanoma lines (F001TU-3 and -4) and 20 separate tumor-infiltrating lymphocyte cultures were generated from a fine needle aspirate of a metastatic lesion progressing after initial response to vaccination. Both F001TU did not express gp100 and were not recognized by C1-35. Loss of gp100 by F001TU correlated with a marked reduction of gp100 expression in the same metastatic lesion compared with prevaccination. Thus, ineffectiveness of C1-35 and tumor progression could be best explained by loss of target Ag expression. Interestingly, 12 of 20 tumor-infiltrating lymphocyte cultures recognized F001TU, but none demonstrated g209/g209-2M reactivity, suggesting a functional dissociation between systemic and local immune response. This study suggests that vaccination effects must be analyzed in the target tissue, rather than in the systemic circulation alone. *The Journal of Immunology*, 1998, 161: 4183–4194.

The identification of melanoma-associated Ags (MAA)[2] and their respective CTL epitopes has raised interest in peptide-based vaccination approaches (1). Among MAA, MART-1/Melan-A and gp100/Pmel 17 (for which the respective CTL epitopes, $MART\text{-}1_{27-35}$ (2) and $gp100_{209-217}$ (g209) (3), have been identified) have been noted to effectively induce CTL reactivity in vitro (4–6). In addition, clinical studies have shown that vaccination with $MART\text{-}1_{27-35}$ and g209 can powerfully enhance specific CTL reactivity in PBMC (7–9). However, the systemic CTL response to the vaccine most of the time does not correspond to clinical regression.

Although the clinical response remains the ultimate therapeutic goal, it is a parameter of little value for the identification of the reason for the most common therapeutic failures. Evaluation of systemic CTL reactivity is generally equated to the level of immune competence toward a certain epitope, and as a consequence is used for the assessment of the effects of a vaccination protocol (10). Assessment of competence toward an immunogen, while yielding an accurate view of the systemic immune response to a vaccine, may not provide sufficient information regarding target/host interactions at the site in which they are likely to occur. In fact, clinical response, including complete responses, has been reported in the context of MAGE-3 peptide vaccination without stimulation of detectable CTL activity at the systemic level (Thierry Boon, personal communication).

The development of peptide-based vaccination protocols for the immunotherapy of melanoma has given us the unique opportunity of comparing systemic T cell responses to a vaccine with localization and status of activation of the same T cells in the target organ.

We therefore wanted to establish a strategy suited for the analysis of CTL response to vaccination at the tumor site. Utilizing functional assays and TCR β-chain analysis, we studied the immune response of a melanoma patient after four cycles of vaccination with g209-2M peptide, a single residue variant of $gp100_{209-217}$ identified as one of the immunodominant HLA-A*0201-restricted CTL epitopes of gp100 (3, 8, 11). An in-depth analysis of T cell reactivity was undertaken in the peripheral circulation and at the tumor site, which revealed a functional dissociation between local and systemic immune response during anti-melanoma vaccination.

Materials and Methods

Cell lines

The melanoma cell lines 624.38 (HLA-A*0201/0301, B*1402/−, Cw*0702/0802) and 624.28 (HLA-A*0301/−, B*1402/−, Cw*0702/0802) were generated by limiting dilution from a metastatic lesion (12). The cell lines 888-MEL (HLA-A*01/2402, B*52/55, Cw*0102/1201) and 1102-MEL (HLA-A*0201/24, B*55/62, Cw*03/−) were derived from other metastatic melanoma lesions. SK23 MEL (HLA-A*0101/0201, B*0702/0801, Cw*0702/0702) and A375 MEL (HLA-A*01/0201, B*17/−, Cw*06/−) melanoma cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). All cell lines were maintained in complete medium (CM) consisting of RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10 mM HEPES buffer, 100 U/ml penicillin-streptomycin (Biofluids), 10 μg/ml Ciprofloxacin (Bayer, West Haven, CT), 0.03% L-glutamine (Biofluids), 0.5 mg/ml amphotericin B (Biotluids), and 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabusas, CA). T2 (ATCC), a cell line defective of endogenous processing and expressing HLA-A*0201 (13), was used to test CTL specificity

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[2] Abbreviations used in this paper: MAA, melanoma-associated antigen; CDR, complementarity-determining region; CM, complete medium; DHDA, direct heteroduplex analysis; FNA, fine needle aspirate; rVV, recombinant vaccinia virus; TIL, tumor-infiltrating lymphocyte.

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toward HLA-A*0201-restricted epitopes. 1520 TIL was expanded with IL-2 from a metastatic lesion of an HLA-A*0201 melanoma patient not previously exposed to vaccination. 1520 TIL naturally recognizes g209.

Peptides

All peptides were produced by solid-phase synthesis technique. The same $gp100_{209-217}$ (g209) (ITDQVPFSV; Chiron Mimotopes Peptide Systems, San Diego, CA), $gp100_{209-217(2M)}$ (g209-2M) (IMDQVPFSV; Chiron Mimotopes), and $MART\text{-}1_{27-35}$ (AAGIGILTV; Peptide Technologies, Gaithersburg, MD) produced for clinical use were used for the in vitro sensitization assays. The residue 2 (T to M) substitution in g209-2M enhances binding to HLA-A*0201, efficiency of T cell induction in vitro (11), and was, for these reasons, preferred to the wild type for vaccination.

HLA typing

HLA class I and II were determined on PBL or tumor cell lines using sequence-specific primer PCR (14). PCR was also used for molecular subtyping of HLA-A2 (15). When necessary, the identity of some HLA alleles was determined conclusively by sequencing of cDNA.

FACS analysis and immunocytochemistry

Cell surface expression of HLA and other surface Ags (CD8, CD4) was determined by flow cytometry. Intracellular staining for the detection of MAA was performed by fixing cells in 200 μl of acetone for 10 min at room temperature before staining with the primary Ab (16). The following mAbs were used: W6/32 (Sera Labs, Westbury, NY) specific for a monomorphic determinant of the HLA class I heavy chain (17); IVA-12 (ATCC) for HLA class II; KS-1 (18) for HLA-A2, (FITC) anti-human CD8, and (FITC) anti-human CD4 (PharMingen, San Diego, CA); and anti-MART-1/Melan-A murine IgG2b (M2-7C10) (16, 19) and anti-Pmel17/gp100 mAbHMB45 (Enzo Diagnostics, Farmingdale, NY). Cytospin preparations of sequentially obtained FNA material were fixed in acetone and stained with the same mAbs used for the FACS analysis, with the exception of HMB45 (Biogenex, San Ramon, CA). For secondary staining, biotinylated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used, followed by avidin-biotin-peroxidase (Vectasin Elite Kit; Vector Laboratories, Burlingame, CA) (16).

Preparation of dendritic cells (DC) and in vitro CTL cultures

Autologous DC utilized for in vitro sensitization of $CD8^+$ T cells were prepared as previously described (20). PBMC were separated from blood by centrifugation on a Ficoll-Hypaque gradient, incubated for 3 h at 37°C, and, after removal of nonadherent cells, cultured for 5 days in CM with 1000 IU/ml IL-4 (PeproTech, Rocky Hill, NJ) and 1000 IU/ml GM-CSF (PeproTech). On day 5, detached DC were harvested and used for stimulation either by peptide pulsing (1 μg/ml g209/DC or g209-2M/DC) or by infection with recombinant vaccinia virus (rVV-MART/DC) (Therion Biologics, Cambridge, MA). $CD8^+$ cells were isolated from PBMC by positive separation (Dynabeads; Dynal, Lake Success, NY). A total of 4×10^6 $CD8^+$ cells/well was cultured in a 24-well plate with 1×10^6 DC. After 24 h and every 2 days thereafter, 300 IU/ml IL-2 was added to the cultures. After 7 days, the cultures were restimulated with rVV-MART-1/DC, g209/DC, or g209-2M/DC and maintained in IL-2 for another week. On days 7 and 14, the cultures were tested for Ag recognition.

Cloning of CTL by limiting dilution

After the second testing for MAA recognition (day 14 of culture), CTL cultures were plated at 100, 10, and 1 cell/well ratio in 96-well round-bottom plates with 5×10^4 irradiated (50 Gy) donor PBMC and 1×10^4 irradiated (100 Gy) EBV-B cells in 200 μl CM supplemented with 30 ng/ml OKT3. After 1 day and every 2 to 3 days thereafter, 300 IU/ml IL-2 was added for 14 to 21 days. Clones were then tested for MAA reactivity. MAA-specific clones were restimulated and expanded in T25 flasks (Costar, Cambridge, MA) with 2.5×10^7 donor PBMC and 5×10^6 1088-EBV-B cells in 25 ml CM with 30 ng/ml OKT3 and IL-2. After in vitro expansion, the cultures were retested for specificity and analyzed for clonality by TCR β PCR, directed heteroduplex analysis (DHDA), and sequencing.

In vitro expansion of TIL and autologous tumor from FNA

Using a 23-gauge needle, cells were aspirated from a metastatic lesion of patient F001 and plated immediately in CM. For TIL expansion, total cells were counted and plated in 24-well plates (of 4×10^6 cells/well) in the presence of 6000 IU/ml IL-2. After 2 wk, the cultures were further expanded in T25 flasks. For expansion of tumor cells, culture conditions were identical to TIL cultures, with the exception of IL-2. The F001TU autologous cell lines were totally HLA class I and II matched to the phenotype of patient F001 and had electron microscopy and karyotyping characteristics consistent with malignant melanoma.

Assessment of Ag recognition by CTL and TIL

IFN-γ release assay. A total of 1×10^5 effector cells was plated with 5×10^4 stimulator cells in 96-well round-bottom plates in 200 μl CM. After 24-h incubation at 37°C, the plates were centrifuged and the supernatant was harvested for analysis by ELISA (Endogen, Cambridge, MA). IFN-γ is reported as pg/ml IFN-γ secreted by 5×10^4 effector cells in 24 h.

Calcein-AM fluorescent cytotoxicity assay. A total of 10^6 target cells/well was incubated with 15 μl Calcein-AM (Molecular Probes, Eugene, OR) for fluorescent labeling. After 30 min, all targets were washed three times in CM and plated in triplicate in 96-well flat-bottom plates at 3000 target cells/100 μl. Effector cells were harvested and added to the target cells at E:T ratios of 10:1, 2.5:1, and 0.625:1 in 100 μl CM. The plates were centrifuged at 500 rpm for 3 min. After 3 h at 37°C, 5 μl of FluoroQuench (One Lambda, Canoga Park, CA) was added to each well to extinguish background fluorescence. The plates were centrifuged, incubated for an additional 60 min, and then scanned on a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA). Fluorescence was quantified using ImageQuant software (Molecular Dynamics). Lysis was calculated using the following formula: (1 − [experimental fluorescence − background fluorescence]/[target only fluorescence − background fluorescence]) × 100.

RNA isolation and cDNA synthesis

For RNA isolation, cells were either taken directly from culture or, if frozen, after overnight culture to allow recovering of physiologic cell metabolism. RNeasy mini or midi kit (Qiagen, Santa Clarita, CA) was used for all RNA isolations. The RNA was eluted with water and stored at −70°C. For cDNA synthesis, about 1 μg of total RNA was transcribed with the SuperScript preamplification system (Life Technologies, Gaithersburg, MD) using the oligo(dT) primer. cDNA was eluted and stored at −25°C.

TCR Vβ PCR and clone-specific PCR

A set of 35 primers was selected to amplify 45 functional Vβ. Each primer mix was composed of 10 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 1.25 U AmpliTaq Gold, 0.5 μl cDNA, 0.5 μM Vβ primer, 0.5 μM TC-1 constant region primer (AYACCAGTGTGGCCTTTT), and water up to 20 μl final reaction volume. A total of 10 μl of light mineral oil covered the reaction mixture, and PCR was run using the following protocol: initial activation of the enzyme at 94°C for 9 min; 10 high-stringency cycles of 94°C for 30-s denaturation, 65°C for 1-min annealing, and 72°C for 1-min elongation; 20 low-stringency cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 10 min. The following 35 primers were used for the Vβ region: TV2 CGAGTTTCTGGTTTCCTTTT for Vβ22s1; TV3-1 ATTTCTGAAGATAATGTTTAGC for Vβ9s1; TV4-1 GAAAGCTAAGAAGCCACCG for Vβ7s1; TV4-2/3 TACAAG CAAAGTGCTAAGAAGC for Vβ7s2, 7s3; TV5-1 GCCTTCAGTTC CTCTTTGA for Vβ5s1; TV5-4/5/6 GGCCCCAGTTTATCTTTC for Vβ5s2, 5s3, 5s6; TV5-8 TCCAGTTCCTCCTTTGGTATG for Vβ5s4; TV6-1 TGGGACTGAGGCTGATTT for Vβ13s3; TV6-2/3/5 GGCT GAGGCTGATTCATTAC for Vβ13s1, 13s2; TV6-4 GGCTAAGGCT CATCCATTAT for Vβ13s5; TV6-6 GGCTGAAGCTGATTTATTAT for Vβ13s6; TV7-2/3 GAGTTTTTAATTTACTTCCAAGGCA for Vβ6s1, 6s5; TV7-6/7/9 CCCAGAGTTTCTGACTTACTTC for Vβ6s3, 6s4, 6s6; TV7-8 GGCCAGAGTTTCTGACTTATT for Vβ6s2; TV9 CCTCCAGT-TCCTCATTCAG for Vβ1s1; TV10-1/3 GGCTGAGGCTGATCCATTAC for Vβ12s1, 12s2; TV10-2 CATGGGCTGAGGCTGATCTA for Vβ12s3; TV11-1 GAGCTTCTGGTTCAATTTCA for Vβ21s1; TV11-2 CCAAAGCTTCTGATTCAGTT for Vβ21s3; TV11-3 GAGCTTCTGAT TCGATATGAGA for Vβ21s2; TV12-3/4 GGACTGGAGTTGCTCATTT for Vβ8s1, 8s2; TV12-5 CAGACAGACCATGATGCAA for Vβ8s3; TV13 CCCAGTTCCTCATTTCGTT for Vβ23s1; TV14 TCGACGTGT TATGGGAAA for Vβ16s1; TV15 CAAAGCTGCTGTTCCACTACTA for Vβ24s1; TV16 GGTCCTGAAAAACGAGTTCAAG for Vβ25s1; TV18 GGTCTGAAATTCATGGTTTATCT for Vβ18s1; TV19 GACAG GACCCAGGGCAAG for Vβ17s1; TV20-1 ATGCTGATGGCAACT TCCA for Vβ2s1; TV24-1 CCTACGGTTGATCTATTACTCCTT for Vβ15s1; TV25-1 CTACACCTCATCCACTATTCCTA for Vβ11s1; TV27 GGGCTTAAGGCAGATCTACT for Vβ14s1; TV28 GGGCTACG GCTGATCTATTTC for Vβ3s1; TV29-1 CACTGATCGCAACTGCAA for Vβ4s1; and TV30 CCTCCAGCTGCTCTTCTA for Vβ20s1.

After PCR, 6 μl of the product and 3 μl of bromphenol blue-loading buffer were mixed and run on a 1% agarose gel for 45 min at 150 V. The gel was stained with Vistra Green (Amersham Life Science, Arlington

Heights, IL) 1/10,000 dilution in 1× TBE for 50 min and analyzed on a FluorImager 595.

Directed heteroduplex analysis (DHDA)

Vβ-specific DHDA was established by a modification of a previously described technique (21). From a donor $CD8^+$ cell pool, a TCR with Vβ6s2 was amplified using TV7-8 and TC-1. The PCR product was cloned into pCR2.1 plasmid (TA-cloning kit; Invitrogen, San Diego, CA) and sequenced to ensure the correct Vβ. From this reference sequence and from C1-35, probes were generated using 6-carboxyfluorescein-labeled TC-1 primer. PCR was performed to obtain the fragments of the samples and the probes for DHDA. To chelate the Mg, 0.2 × vol of 25 mM EDTA was added to the probe. The heteroduplex generation was done with 2 μl of the sample PCR product and 2 μl of the probe by denaturating at 96°C for 5 min, then cooling rapidly to 50°C, and renaturating at this temperature for 45 min. The samples were mixed with 2 μl of loading buffer/Prism Genescan-500 TAMRA size marker (ABI Perkin-Elmer, Foster City, CA) and loaded on a native gel (4% bis-acrylamide:acrylamide 1:19). The gel was run in an ABI Sequencer 377 at 50°C for 2 h. The result was analyzed using the GeneScan software (ABI Perkin-Elmer).

Sequencing of the TCR β-chain

The TCR β-chain of C1-35 was amplified with TC-1 and TV7-8 using Pfu polymerase, and the product was cloned into the TA vector. Sequences from both directions were obtained from five bacterial clones using the ABI PRISM Dye Primer kit and the ABI PRISM 377 DNA Sequencer. The sequences were analyzed with the ABI software, Sequence Analysis and Sequence Navigator. The Vβ6s2 PCR products of the other 16 cultures that were analyzed for clonal identity were sequenced directly from PCR products using ABI PRISM Dye Terminator kit. The sequences of the H.3-1 and F001TIL-9 were also obtained directly from PCR products using TV12-3/4 and TV4-2/3, respectively.

Direct PCR and nested PCR with clone-specific CDR3 primers

Clone-specific TCR β-chain primers were derived from the sequence of the CDR3 region of C1-35 (C35-2 CAT CGCCCCGCTCCCCCCAG) and F001TIL-9 (T9-2 AAGAACTGCTCATTGTAGTAAGTA). The direct PCR used the primers TV7-8 and C35-2 for the amplification of the C1-35 TCR β, and TV4-2/3 and T9-2 for the amplification of F001TIL-9 TCR β. The reaction mixture was composed of 10× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 1.25 U AmpliTaq Gold, 0.5 μl cDNA, 1 μl each primer (10 μM), and water up to 20 μl. A total of 10 μl of light mineral oil covered the reaction mixtures, and PCR was run as described for TCR β PCR. A total of 6 μl of the PCR product and 3 μl of bromphenol blue-loading buffer were mixed and run on a 1% agarose gel for 45 min at 150 V. The gel was stained and analyzed as described for TCR β PCR. To control for the relative amount of T cells, a Cα control was run with the primers TAC-F1 (ATATCCAGAACCCTGACCTGC) and TCA-R1 (GCTTTC TCGACCAGCTTGACATC). For the nested PCR, an amplification using the external primer RTV7-2 (ATCACACAGGRGCTGGAGT) for C1-35 and RTV4-2 (ATGGAAACGGGAGTTACG) for F001TIL-9 was performed before the clone-specific amplification. After the first amplification, the PCR product was diluted 1/5 with water, and from the dilution 1 μl used for the second amplification. The second amplification was done as described above for the direct PCR.

Results

Patient's history

A single lesion from a patient with metastatic melanoma (F001) undergoing vaccination with g209-2M peptide was followed. The protocol was approved by Institutional Review Board of National Cancer Institute. The patient's HLA class I phenotype determined by sequencing was A*0201/0301, B*0702/0801, Cw*0701/0702. After two vaccinations with g209-2M in combination with IL-12, an initial reduction of the tumor mass was observed on physical examination and radiographic evaluation. PBMC were collected before and after vaccination. Within 1 mo, the tumor mass became unresponsive to further treatment and progressed in size. At this point, a FNA of the mass was performed for analysis of tumor cells and TIL.

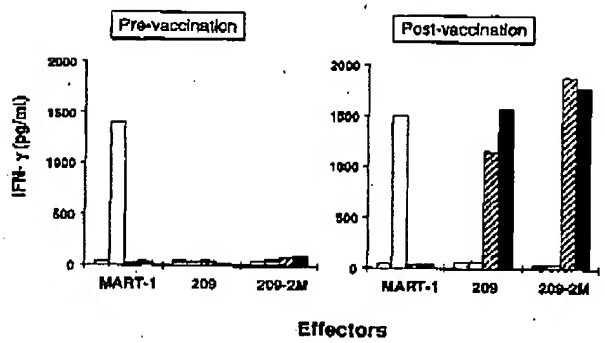


FIGURE 1. MAA-specific reactivity of CTL bulk cultures from pre- and postvaccination PBMC. After 2 wk, CTL bulk cultures were tested for IFN-γ release in response to T2 alone (gray bars) or T2 pulsed with $MART\text{-}1_{27-35}$ (open bars), g209 (hatched bars), or g209-2M (filled bars). MART-1 effectors, CTL culture stimulated with rVV-MART-1-infected autologous DC; g209 effectors, CTL culture stimulated with g209-pulsed autologous DC; g209-2M effectors, CTL culture stimulated with g209-2M-pulsed autologous DC. These results are representative of three independent experiments.

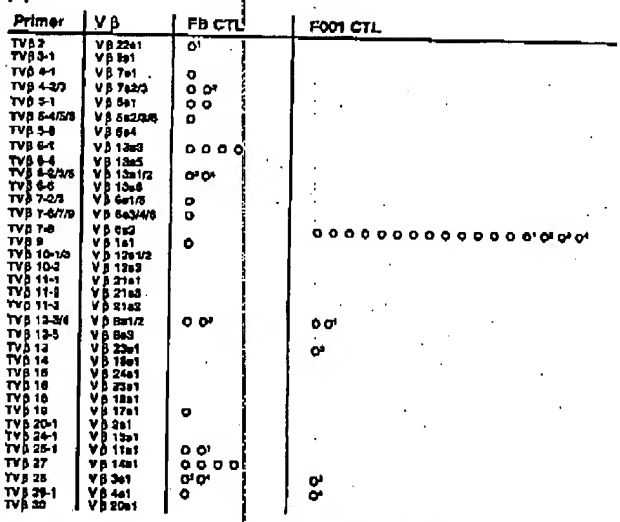
In vitro reactivity to g209 and g209-2M is observed only in postvaccination CTL cultures

Cultures of $CD8^+$ cells from pre- and postvaccination PBMC were generated using autologous DC either infected with rVV-MAA or loaded with 1 μg/ml g209 or g209-2M. When tested for MAA recognition after 2 wk in culture, no reactivity to g209 or g209-2M could be detected in prevaccination cultures, whereas postvaccination cultures demonstrated a strong sensitization against the natural as well as the modified gp100 epitope (Fig. 1). Although the patient had not been exposed to exogenous administration of MART-1, MART-1 reactivity could be equally observed in pre- and postvaccination cultures. This is not uncommon, as naturally occurring MART-1-specific reactivity can be readily detected in the peripheral circulation of HLA-A*0201-expressing melanoma patients (4).

A predominant CTL clone recognizing g209 and g209-2M is observed in postvaccination CTL cultures

CTL clones were raised by limiting dilution from postvaccination CTL cultures induced in vitro. Cloning efficiency was about 100, 30, and 10% for the 100, 10, and 1 cell/well plates, respectively. Proliferating wells were tested for recognition of g209/g209-2M. From all g209/g209-2M-reactive 10 and 1 cell/well cultures, 25 clones were randomly selected for further expansion. After 2 wk of expansion, 18 of 25 cultures induced with g209-2M-pulsed DC maintained their specific reactivity. To assess the clonality of these 18 cultures, TCR β PCR was performed for a general overview: 17 of the 18 cultures showed a common strong band in Vβ6s2 (Fig. 2*A*). Among these, all 12 cultures expanded from the 10 cell/well plates demonstrated additional weaker bands specific for other Vβ families, whereas no other bands were observed in the five cultures from 1 cell/well plates, suggesting the purity of these clones. To determine whether the Vβ6s2 bands represented the same TCR or originated from a different TCR utilizing the same Vβ, DHDA was performed with the Vβ6s2 PCR products from the 17 samples (Fig. 2*B*). When an irrelevant TCR β with Vβ6s2 from a healthy donor was utilized as probe, all 17 samples showed a single heteroduplex band that migrated with identical delay relative to the homoduplex band. This indicated that the samples contained only one TCR and that the mismatches between the sample TCR β and

A



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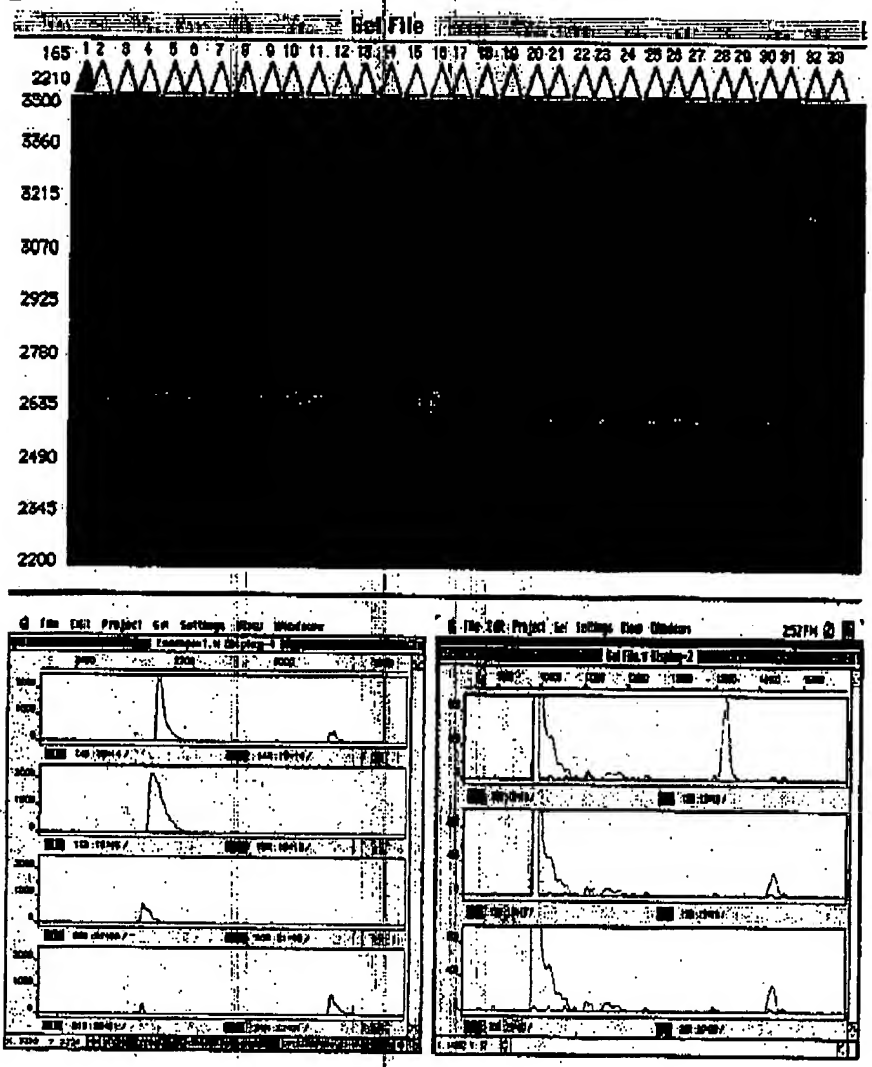

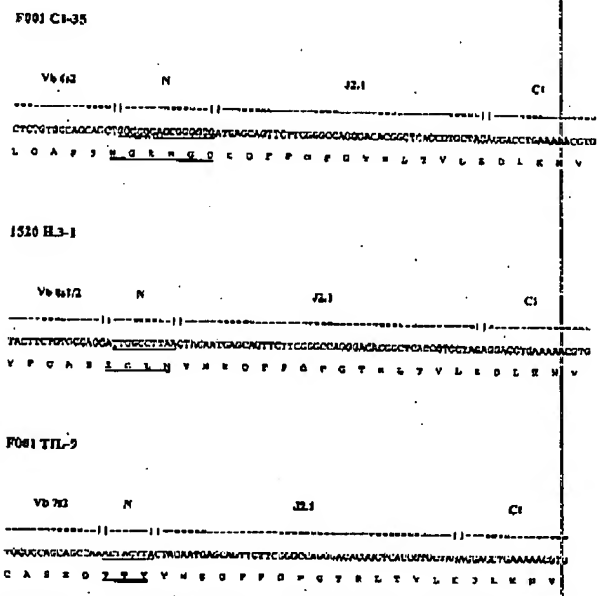

FIGURE 3. Sequences of TCR β-chains around the CDR3. These sequence data are available from GenBank: F001 C1-35 under accession number AF037565, 1520 H.3-1 under AF037566, and F001 TIL-9 under accession number AF037567.

the probe were similar, if not identical, among the clones. To validate the identity of these clones, one of the sample TCR β was labeled and used as probe against all other samples. With this probe, only a homoduplex was detected in all samples. These data strongly suggested identity of TCR β among the clones. The identity of the 17 TCR β-chains was verified additionally by sequencing each of them (Fig. 3). Thus, a predominant g209/g209-2M-specific clone expanded by the g209-2M vaccine was identified. For functional studies, one clone (C1-35) was selected as representative of the g209/g209-2M-reactive CTL population. The 18th clone (C10-80) included in the TCR β PCR analysis showed only a faint Vβ6s2 band and two strong bands in other Vβ.

*The predominant clone C1-35 recognizes naturally processed g209 on HLA-A*0201-matched melanoma cell lines, and demonstrates a lower avidity of TCR epitope interactions compared with a naturally occurring g209-specific TIL clone*

C1-35 was able to lyse HLA-A*0201, gp100-expressing melanoma cell lines, including 624.38 MEL matched at three alleles (HLAA*0201/0301, -B*1402, -Cw*0702/0802), 526 MEL and 1102 MEL matched only at HLA-A*0201, but could not lyse the totally mismatched 888 MEL. Furthermore, the HLA-A*0201-expressing, gp100-negative F001TU-3 autologous cell line was insensitive to lysis by C1-35 (Fig. 4A). Upon stimulation with other HLA-A*0201-matched melanoma cell lines, C1-35 was noted to secrete IFN-γ only in response to some targets characterized by high expression of HLA-A*0201 and gp100 (Fig. 4B). As the ability of C1-35 to release IFN-γ when stimulated with HLA-A*0201-matched tumor lines could be dependent upon the avidity of TCR/epitope interactions (22), epitope density requirements for IFN-γ release were compared between C1-35 and a clone (H.3-1) from 1520 TIL. This TIL was derived from a metastatic lesion of a different patient not previously exposed to epitope-specific vaccination, and is characterized by high avidity interactions with gp100/HLA-A*0201-expressing melanoma cells. Loading T2 cells with decremental g209 or g209-2M doses, the epitope density needed to activate IFN-γ release by the two clones was compared. Although both were sensitive to stimulation, a 50- and a 100-fold lower concentration of g209 and g209-2M, respectively, were found to stimulate comparable amounts of IFN-γ release by H.3-1 compared with C1-35 (Fig. 4C). Apparently, the g209-2M vaccination had induced and expanded in vivo a CTL clone with an avidity for its target that was lower than the one observed in a naturally occurring g209-specific TIL clone. As a consequence, C1-35 could recognize some, although not all, gp100-expressing, HLA-A*0201-matched melanoma cell lines recognized by H.3-1.

Development of TIL and autologous tumor cells by expansion of FNA material

Progressive tumor growth could have been due to lack of CTL localization at the tumor site, despite the presence of g209-specific CTL in the peripheral blood. To examine molecularly and functionally whether C1-35 was detectable at the time of progression, we performed a FNA of the growing metastasis at the moment of clinical progression. From the FNA material, expansion of TIL (IL-2, 6000 IU/ml added to CM) and autologous tumor was attempted. Twenty separate TIL cultures (F001TIL-1 to -20) and two tumor cell lines (F001TU-3 and -4) were generated.

TCR β-chain repertoire in PBMC, immune CTL, and TIL

To characterize the TCR repertoire in the PBMC, CTL cultures, and TIL, TCR β PCR with 35 Vβ-specific primers for 45 functional Vβ was performed (Fig. 5). $CD8^+$ T cells from pre- and postvaccination PBMC showed a broad usage of Vβ with bands in 23 of 35 reactions. Postvaccination g209 CTL cultures showed little variation compared with the PBMC with losses of bands in Vβ8s3 (*lane 7*), Vβ13s3 (*lane 29*), Vβ13s5 (*lane 30*), and Vβ1s1 (*lane 35*), and relatively denser bands in Vβ21s3 (*lane 4*), Vβ15s1 (*lane 16*), and Vβ6s3/6s4/6s6 (*lane 33*). Postvaccination g209-2M CTL cultures presented a denser band corresponding to the Vβ6s2 (*lane 34*) chain utilized by the majority of the CTL clones expanded from the bulk culture, suggesting that C1-35 TCR expansion was not an artifact related to the cloning conditions. C1-35,

FIGURE 2. Analysis of clonality by TCR β PCR and DHDA of 18 cultures. *A*, TCR β PCR for 18 g209-reactive CTL clones generated by limiting dilution of g209-2M-induced postvaccination CTL cultures from patient F001. Each circle represents a strong band; weak bands are not included. Cultures with two strong bands are indicated with superscript numbers; circles with the same number belong to the same culture. FB CTL are limiting dilution cultures raised similarly to F001 CTL from PBL of a melanoma patient who had never received Ag-specific vaccination. In this case, the TCR β usage of CTL specific for the same epitope was highly polyclonal. *B*, DHDA was done with the predominant TCR Vβ6s2 PCR products of 17 cultures (only 14 of 17 shown) using an unrelated TCR Vβ6s2 with a different CDR3 (*I*) and C1-35 (*II*). *I, Lanes 2–15*, 14 samples + labeled probe; *lane 16*, unlabeled probe + labeled probe; *lane 17*, labeled probe alone. *II, Lanes 18–31*, 14 samples + labeled C1-35; *lane 32*, unlabeled probe + labeled C1-35; *lane 33*, labeled C1-35 alone. The two panels of electrophorograms below the gel display results of computer analyses for individual lanes. In the *left panel, lanes 15, 16, 31,* and *32* of the gel above are shown. The *right panel* exemplifies how different clones presenting identical bands in the TCR β PCR can be distinguished by DHDA. Three of four FB CTL clones with Vβ13s3 (see *A*) were analyzed. The heteroduplexes of two (*middle* and *bottom*) migrated identically, but one (*top*) migrated significantly faster, proving this clone to carry a different TCR β.

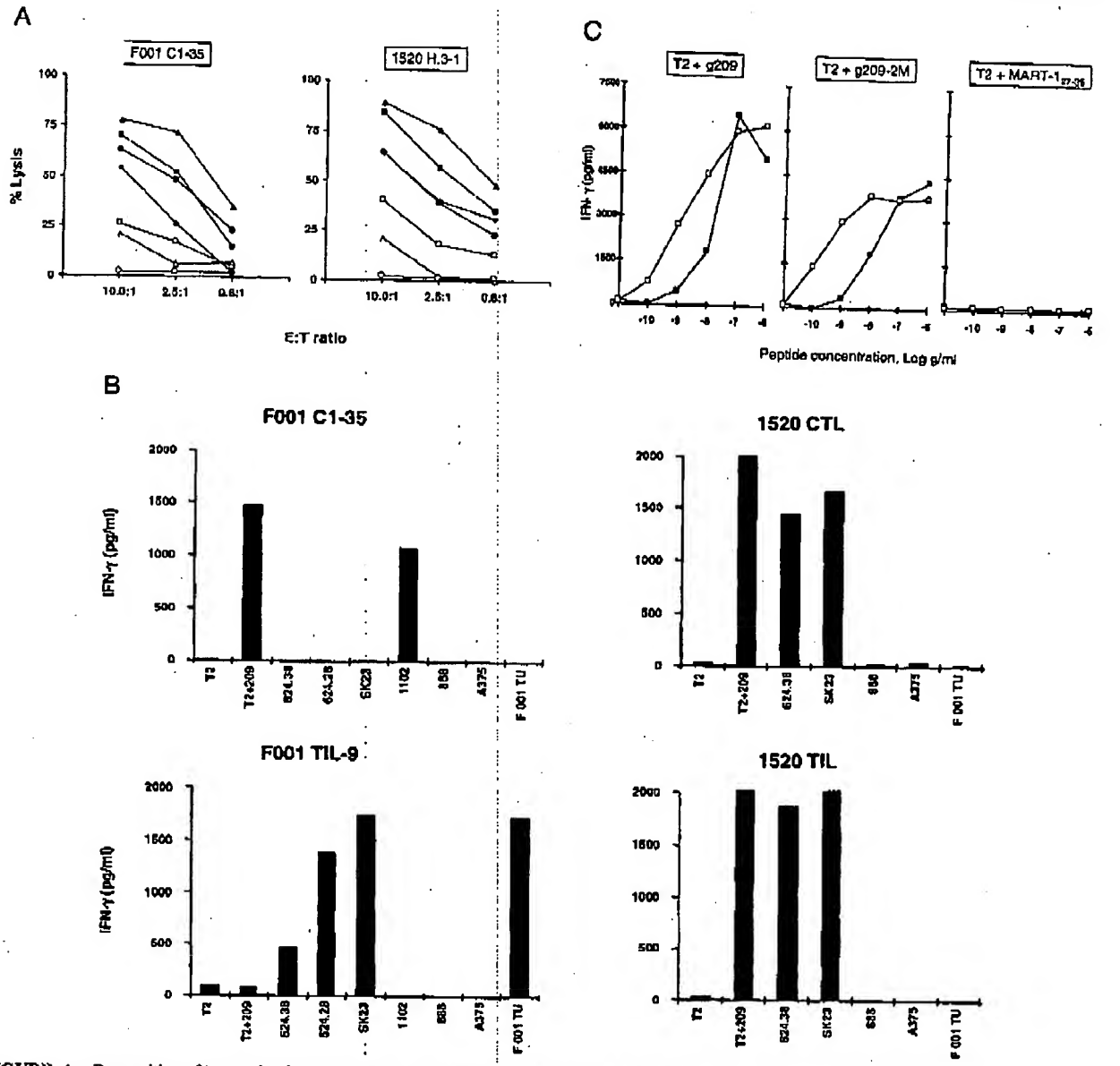

FIGURE 4. Recognition of tumors by C1-35 and F001TIL-9 and functional avidity analysis. *A*, Calcein-AM cytotoxicity assay of C1-35 compared with H.3-1, a clone from a naturally occurring TIL culture (1520 TIL) recognizing g209/g209-2M. Lysis of Calcein-AM-labeled targets was measured at the E:T ratios of 10:1, 2.5:1, and 0.6:1. Targets included T2 + MART-1_{27-35} (□), T2 + g209 (■); the HLA-A*0201+/gp100+ 1102 MEL (●), 526 MEL (♦), 624.38 MEL (▲) lines; the HLA-A*0201−/gp100+ 888 MEL (△) and the autologous gp100− F001TU-3 (○). *B*, IFN-γ release assay of C1-35 and TIL-9 from patient F001 compared with 1520 CTL and 1520 TIL. Targets included the HLA-Cw*0702-matched lines 624.38 MEL, 624.28 MEL, SK23 MEL, and autologous F001; the HLA-A*0201-matched, but not Cw*0702-matched, 1102 MEL and A 375 MEL; and the totally unmatched 888 MEL. *C*, IFN-γ release by C1-35 (■) and H.3-1 (□) after incubation with T2 cells pulsed with 10-fold dilutions of MAA peptides ranging from 10 to 0.0001 μg/ml. Results are representative of three independent experiments.

representative of all other related clones, showed a dominant band corresponding to Vβ6s2 (*lane 34*). Faint bands for Vβ13s1/13s2 (*lane 9*), Vβ17s1 (*lane 14*), Vβ14s1 (*lane 18*), and Vβ3s1 (*lane 19*) were not detected with ethidium bromide staining, but only with the more sensitive Vistra Green staining, and were regarded as trace contamination. C10-80, the only clone of 18 that did not present a dominant Vβ6s2 (only a weak band in *lane 34*), was found to have predominantly Vβ8s1/8s2 (*lane 6*) and Vβ22s1 (*lane 15*). The FNA, which represents the local TCR β repertoire of the lesion, displayed bands for Vβ13s1/13s2 (*lane 9*), Vβ17s1 (*lane 14*), Vβ14s1 (*lane 18*), Vβ4s1 (*lane 20*), Vβ7s1 (*lane 24*), Vβ7s2/7s3 (*lane 25*), and Vβ6s2 (*lane 34*). Due to low amount of RNA from the FNA material, the intensity of the bands was much weaker, and it cannot be ruled out that other Vβs were missed for technical reasons. When F001TIL-9 was tested, it appeared to be almost pure, with only a faint band for Vβ6s2 (*lane 34*), besides the dominant band for Vβ7s2/7s3 (*lane 25*). Taken together, the monitoring of the TCR β repertoire of different original and in vitro culture samples allowed a

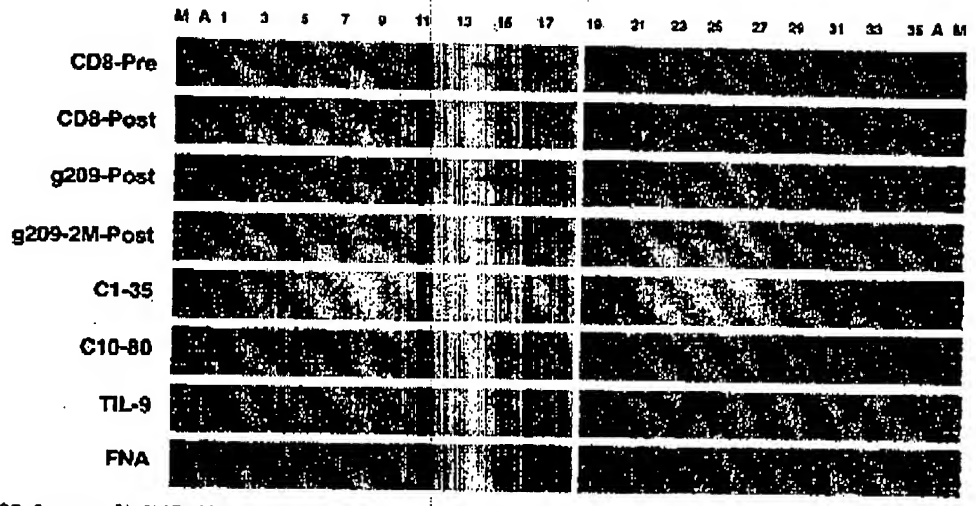

FIGURE 5. TCR β usage of PBMC, CTL cultures, TIL, and FNA. *M*, 100-bp ladder; *A*, β-actin control; *lane 1*, TV10-1/3 (Vβ12s1, 12s2); *lane 2*, TV10-2 (Vβ12s3); *lane 3*, TV11-1 (Vβ21s1); *lane 4*, TV11-2 (Vβ21s3); *Lane 5*, TV11-3 (Vβ21s2); *lane 6*, TV12-3/4 (Vβ8s1, 8s2); *lane 7*, TV12-5 (Vβ8s3); *lane 8*, TV13 (Vβ23s1); *lane 9*, TV6-2/3/5 (Vβ13s1, 13s2); *lane 10*, TV14 (Vβ16s1); *lane 11*, TV15 (Vβ24s1); *lane 12*, TV16 (Vβ25s1); *lane 13*, TV18 (Vβ18s1); *lane 14*, TV19 (Vβ17s1); *lane 15*, TV2 (Vβ22s1); *lane 16*, TV24-1 (Vβ15s1); *lane 17*, TV25-1 (Vβ11s1); *lane 18*, TV27 (Vβ14s1); *lane 19*, TV28 (Vβ3s1); *lane 20*, TV29-1 (Vβ4s1); *lane 21*, TV20-1 (Vβ2s1); *lane 22*, TV3-1 (Vβ9s1); *lane 23*, TV30 (Vβ20s1); *lane 24*, TV4-1 (Vβ7s1); *lane 25*, TV4-2/3 (Vβ7s2, 7s3); *lane 26*, TV5-1 (Vβ5s1); *lane 27*, TV5-4/5/6 (Vβ5s2, 5s3, 5s6); *lane 28*, TV5-8 (Vβ5s4); *lane 29*, TV6-1 (Vβ13s3); *lane 30*, TV6-4 (Vβ13s5); *lane 31*, TV6-6 (Vβ13s6); *lane 32*, TV7-2/3 (Vβ6s1, 6s5); *lane 33*, TV7-6/7/9 (Vβ6s3, 6s4, 6s6); *lane 34*, TV7-8 (Vβ6s2); *lane 35*, TV9 (Vβ1s1).

general overview of the changes and clonality status. For example, based on TCR β PCR alone, cultures such as C10-80 could be identified as not clonal. This method, however, could not provide any information about the composition of a band, nor could it tell whether the same clone was responsible for corresponding bands in different samples. For the former limitation, DHDA and sequencing were applied as described above, and for the latter, clone-specific PCR analysis was performed.

Clone-specific analysis reveals that C1-35 expanded specifically in response to vaccination and localized at tumor site

To assess the presence of clone C1-35 TCR in the different samples, clone-specific PCR was performed using a primer for the CDR3 region of the C1-35 TCR β-chain. The direct PCR clearly detected the clone in the g209-2M bulk culture from which the clone was derived. In addition, weak bands were visible in the postvaccination PBL, g209/g209-2M cultures, FNA, and F001TIL (Fig. 6). To corroborate this result, a nested PCR consisting of a Vβ-specific first amplification and a clone-specific second amplification was performed. The results of the nested PCR confirmed the bands in the postvaccination samples, while all prevaccination samples remained negative. By clone-specific analysis, F001TIL-9 could be detected only in TIL cultures, but not in the FNA from which it was expanded. This sensitivity limitation could not be overcome even by nested PCR (Fig. 6). Several unsuccessful attempts to tailor the PCR conditions to this specific reaction suggested that F001TIL-9 was present in minor proportion in vivo, although was readily sensitive to the proliferative stimulus provided in vitro by high dose IL-2. A panel of C region α-chain (C-α) amplifications was run along with the direct amplification to make semiquantitative assessments of the PCR results. Considering the low signal intensity for the C-α and the stronger signal for clone C1-35 TCR β-chain in FNA compared with the postvaccination $CD8^+$ PBL preparations, the technically inevitable contamination of the FNA with peripheral blood is unlikely to solely account for the C1-35 TCR β signal detected in the FNA. These data suggested that lack of localization of C1-35 at tumor site could not explain the regained tumor growth.

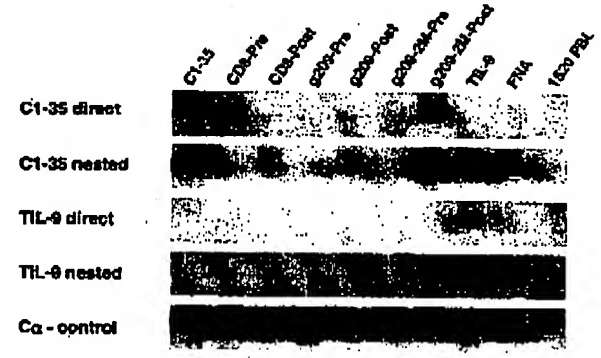

FIGURE 6. Clone-specific analysis by CDR3-specific PCR. C1-35 direct, clone-specific PCR with CDR3 primer specific for C1-35 TCR β; C1-35 nested, nested PCR for C1-35 with first amplification using the constant primer and external Vβ primer and second amplification identical to the direct PCR; TIL-9 direct, clone-specific PCR with CDR3 primer specific for TIL-9 TCR β; TIL-9 nested, nested PCR for TIL-9 with first amplification using constant primer and external Vβ primer and second amplification identical to the direct PCR. Cα control, amplification of Cα fragment to assess the amount of T lymphocytes in the samples. RNA samples were obtained from purified $CD8^+$ preparations from PBMC obtained before (CD8-Pre) and after vaccination (CD8-Post), CTL cultures induced from the same $CD8^+$ preparations with g209-pulsed DC (g209-Pre and g209-Post, respectively), or g209-2M-pulsed DC (g209-2M-Pre and g209-2M-Post, respectively). RNA was also prepared from C1-35 and F001TIL-9 CTL (as controls) from the postvaccination FNA from which the reagents described in this study were obtained (FNA), and from 1520 PBL as a negative control PBL from a different melanoma patient.

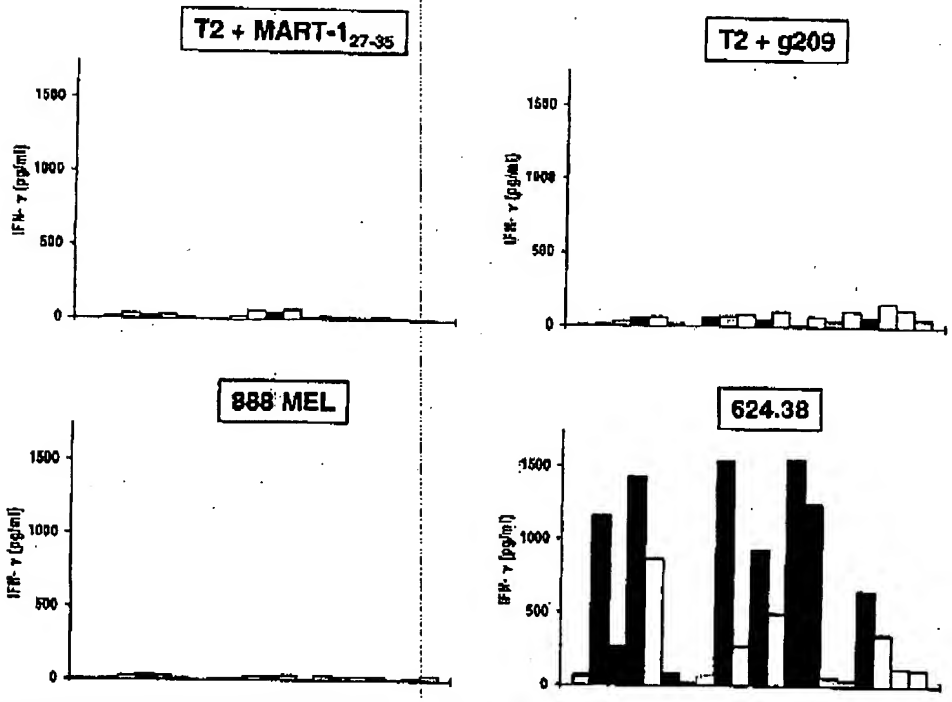

FIGURE 7. Recognition of peptide-pulsed targets and tumors by 20 TIL cultures developed from a FNA. Twenty TIL cultures developed from a FNA obtained from a progressing lesion post-g209-2M vaccination were tested for IFN-γ release in response to MART-1_{27-35} or $_{g209}$-pulsed T2 cells and in response to a HLA-matched melanoma 624.38 MEL (matched at HLA-A*0201,0301 and -Cw*0702). The melanoma cell line 888 (888 MEL) represented a totally HLA class I- and class II-mismatched negative control.

Postvaccination TIL cultures from FNA of the progressing tumor show reactivity to the tumor, but fail to recognize g209/g209-2M

From the FNA material, two autologous tumor cell lines (F001TU-3 and F001TU-4) and 20 separate TIL cultures were generated. The TIL cultures were >95% $CD8^+$ and were tested for recognition of g209 and HLA-matched tumors (Fig. 7). Surprisingly, none of the 20 TIL cultures recognized T2 targets pulsed with either g209 or g209-2M. TIL were also unable to recognize other known HLA-A*0201-associated MAA epitopes, including MART-1_{27-35} (2, 3, 23–25). However, 12 of 20 cultures could recognize autologous as well as other HLA-B*0702- and HLA-Cw*0702-matched melanoma targets, but not the autologous EBV-B nor other matched nonmelanoma cells. To exclude the possibility of a transplantation Ag reaction, HLA typing of TIL was performed and found to be identical to the patient's (A*0201/0301, B*0702/0801, Cw*0701/0702). Attempts to expand TIL populations from the FNA with OKT-3 and feeder cells (as used for cloning of CTL) also failed to generate g209/g209-2M-reactive TIL (data not shown). Thus, C1-35 and F001TIL-9 demonstrated a functional dissociation in epitope specificity between reagents obtained from the peripheral circulation and those obtained from the tumor (Fig. 4*B*).

Analysis of FNA material and of cultured autologous tumor reveals loss of the gp100 Ag after vaccination with g209-2M

As noted in Figure 4*B*, C1-35, 1520 TIL, and its high avidity clone H.3-1 failed to recognize F001TU-3. These findings suggested loss of either HLA-A*0201 or gp100 expression by the autologous tumor. FACS analysis of F001TU-3 and -4 demonstrated loss of gp100 expression and retention of expression of HLA-A*0201 (Fig. 8*A*). Sequencing of F001TU-3 cDNA ruled out mutations of the HLA-A*0201 heavy chain. Because of the possibility that F001TU-3 and -4 had originated from rare cells in the FNA material not representative of the tumor, cytospin preparations from the original FNA were analyzed by immunocytochemistry and compared with FNA material obtained from the same lesion at various time points. This analysis confirmed a marked decrease in gp100-expressing cells postvaccination (Fig. 8*B*), while the expression of HLA-A*0201 remained unchanged. Indeed, while gp100 was detectable in >75% of tumor cells in the prevaccination FNA, less than 5% of cells expressed gp100 postvaccination (Table I). MART-1 expression was not affected by the vaccination, although analysis of F001TU-3 and -4 revealed decreased expression of this MAA. Expression of HLA-A*0201 was similar in all FNA analyzed. Although C1-35 and H.3-1 could not naturally recognize F001TU-3 and -4 (Fig. 4, *A* and *B*), exogenous loading of peptide on F001TU and other HLA-A*0201 melanomas not recognized by C1-35 could stimulate IFN-γ release (Fig. 9). These data suggest that the poor recognition of autologous tumor by C1-35 and H.3-1 was due to inadequate epitope density on the cell surface rather than abnormalities of the HLA-A2 heavy chain or killer inhibitory receptor-HLA interactions (26). Thus, tumor escape from peptide vaccination was associated with severely decreased expression of target Ag by the tumor, which led to proliferation of a cell population not recognizable not only by the intermediate avidity CTL elicited by the vaccination, but also by high affinity CTL effectors.

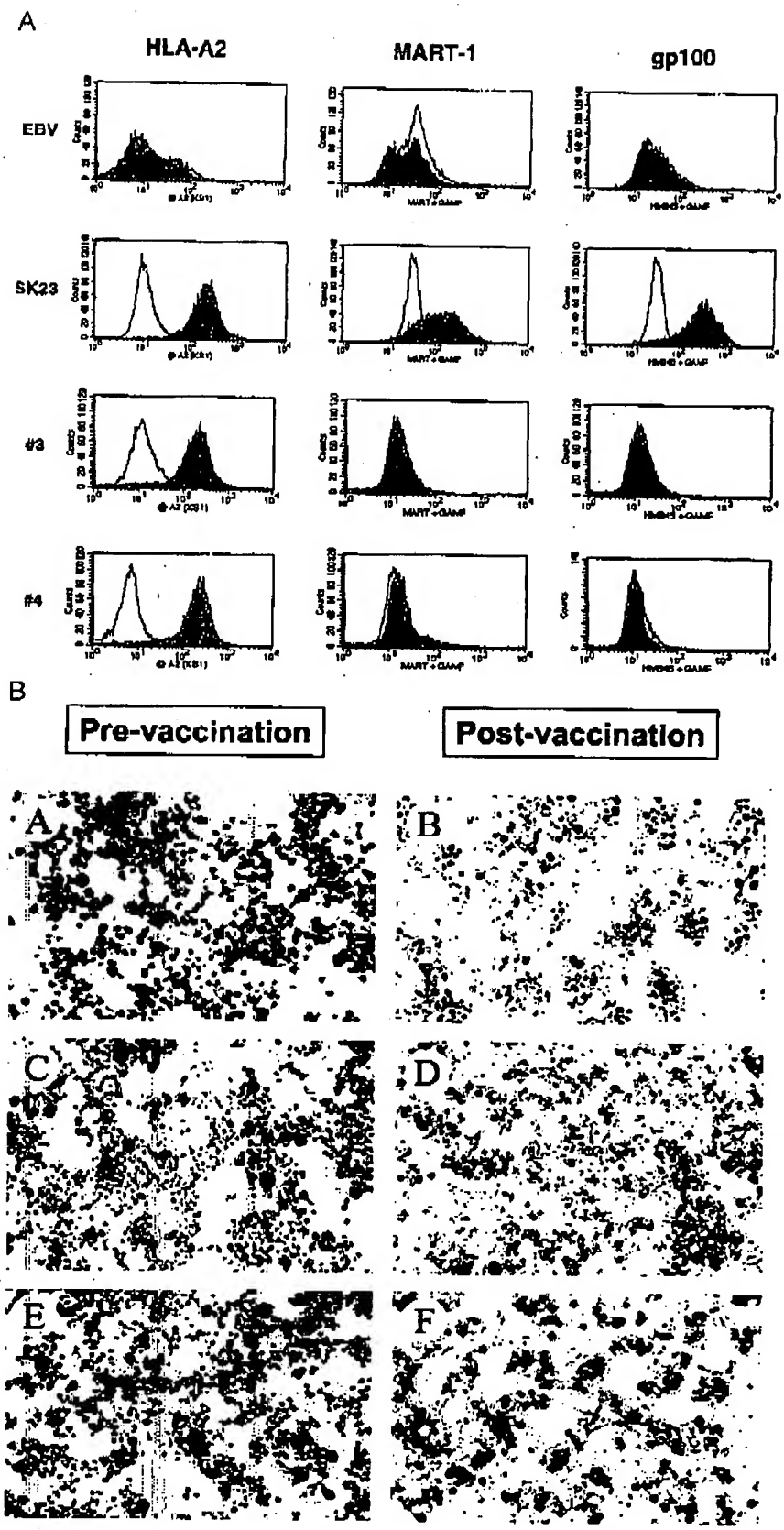

FIGURE 8. *A*, Analysis of HLA-A2 and MAA expression by F001TU-3 and F001TU-4 melanomas. F001TU-3 and F001TU-4 were analyzed by FACS for the expression of HLA-A2, MART-1, and gp100 Ags. Tumor cells were stained with mAbs specific for the HLA-A2 (KS1 (18)), MART-1 (M2-7C10 mAb (19)), and gp100 (HMB-45). The EBV-B cell line 888 and the melanoma cell line SK23 were used as negative and positive controls for the expression of MAA and HLA-A2, respectively. *B*, Immunocytochemical analysis of HLA-A2 and MAA expression in FNA obtained pre- and postvaccination with 209-2M peptide. Cytospin preparations of FNA material obtained from a metastasis pre-g209-2M vaccination (*A*, *C*, *E*) and from the same progressing lesion postvaccination (*B*, *D*, *F*). Cytospins were stained with HMB-45 mAb (Biogenex) for detection of gp100 (*A*, *B*), M2-7C10 mAb (19) for MART-1 (*C*, *D*), and KS-1 (18) for HLA-A2 (*E*, *F*). Positive cells are indicated by the brown chromogen 3,3'-diaminobenzidine. All cells were counterstained with hematoxylin (blue).

Table 1. *Immunocytochemistry of FNA material for MAA and HLA-A2 expression*

Time of FNA	Date	Expression of gp100[a]	MART-1[a]	HLA-A*0201
Prevaccination	1/30/1997	50–75	25–50	Positive
Postvaccination	5/28/1997	<5	50–75	Positive
	6/18/1997	<5	50–75	Positive
	7/11/1997	<5	50–75	Positive

[a] Data are % of staining cells.

Discussion

With the identification of CTL epitopes believed to be clinically relevant, peptide-based vaccinations have been developed. The rationale is to provide proper stimulatory conditions that could adequately turn on an immune system otherwise insensitive to Ags expressed by tumor cells (27). Murine models predict that tumors do not induce spontaneously nor maintain an activated CTL response even after activation of protective T cells by vaccines, and repetitive immunizations are necessary for prolonged effects (28). The primary physiologic effect of vaccination is a systemic enhancement of responsive T cells. The desired secondary effect, however, is the localization of activated CTL at the tumor site to perform their therapeutic functions. We have shown that the s.c. administration of MAA epitopes in IFA induces MAA-specific CTL detectable in the peripheral blood that can recognize naturally processed epitopes on the surface of tumor cells (7, 8). However, by itself, peptide administration rarely leads to tumor regression. Clinical response after vaccination is generally attributed to the ability of activated CTL to localize at tumor site and kill tumor cells. However, the exact mechanism responsible for the more common therapeutic failures is unclear. Analysis of tumor/host interactions at the site of disease may provide such information. Excisional biopsy of tumors and subsequent expansion of TIL/tumor pairs is a useful tool for the analysis of tumor/host interactions at a given point (29). Although useful, this strategy has not yielded conclusive information for three reasons. First, homogeneity among tumors must be assumed to take the excised lesion as representative of other metastases left in vivo for clinical correlation. However, synchronous metastases are often heterogeneous in expression of MAA and HLA (30). Second, the natural course of the tumor cannot be determined after its removal: only a retrospective correlation can be performed between clinical parameters and characteristics of the reagents obtained from the biopsy. Finally, the removal of the tumor excludes comparative studies of the same lesion at different points in time in relation to the natural progression of the disease or in response to immune pressure.

To overcome the limitations posed by excisional biopsy, we suggest following metastases by serial FNA, which allows the evaluation of tumors at various points (16). By following the same lesion serially, heterogeneity among tumors can be avoided as a confounding factor. The ability to expand TIL and autologous tumor from the FNA permits the analysis of CTL localization and function at tumor site. This strategy was tested on a melanoma patient with a metastasis of particular interest: the mass had shrunk after vaccination with g209-2M, suggesting effectiveness of treatment. However, after the initial shrinkage, the lesion became insensitive to further g209-2M vaccines.

The identification of g209/g209-2M-reactive CTL from postvaccination PBMC, in concordance with the lack of C1-35 detection by PCR in prevaccination samples, was an indication of successful systemic induction and expansion of vaccine-specific CTL. The presence of g209-reactive CTL at that time point suggested that tumor progression was not the result of functional deletion of tumor-specific CTL by the vaccine, as suggested by some murine models (31). The predominance of a single clone after vaccination was of interest and contrasted with the capacity observed in naturally occurring CTL, recognizing immunodominant epitopes such as MART-1_{27-35} (32) or EBNA$4_{416-424}$ (33) to maintain a broad TCR repertoire. In fact, we observed, in a second melanoma patient not previously exposed to MAA-specific vaccination, a

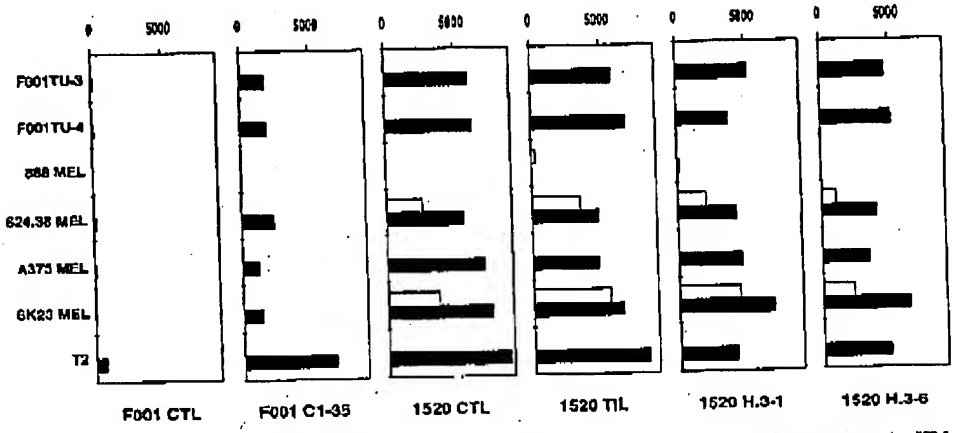

FIGURE 9. Recognition of naturally processed g209-2M by F001 and 1520 CTL. F001 CTL and F001C1-35 were tested by IFN-γ release assay for recognition of the autologous F001TU-3 and F001TU-4; the HLA-A*0201-matched melanomas 624.38 MEL, A375 MEL, SK23 MEL. 624.38 MEL, and SK23 MEL were selected as cell lines with intermediate expression of HLA-A*0201 and gp100. These lines can be killed in cytotoxicity assays, but do not stimulate IFN-γ release by C1-35 (see Fig. 4*B*). The 209-2 M 1520 CTL, 1520 TIL, and the 1520 TIL clones H.3-1 and H.3-6 obtained from a different melanoma patient. 1520 TIL were expanded from a metastasis before vaccination, while the 1520 CTL cultures were induced in vitro by stimulation of PBMC obtained from the same patient post-209-2 M vaccination. T2 cells pulsed with gp209 were also included, as they can efficiently present the relevant peptide and stimulate IFN-γ release by peptide-specific reactive CTL. The melanoma cell line 888 (888 MEL) represented the HLA-A-mismatched negative control (HLA-A1, 24). Filled bars represent IFN-γ release in response to stimulator cells pulsed with g209 (1 μg/ml). Open bars indicate IFN-γ release in response to nonpulsed stimulator cells. Results are representative of three independent experiments.

widely polyclonal, MART-1_{27-35}-specific CTL population characterized by broad Vβ usage.

Several explanations are plausible for the predominance of C1-35, including an artifact of in vitro culture (34), a consequence of the vaccination procedure, or a direct effect of the high affinity interactions between g209-2M and HLA-A*0201 (11). In a functional peptide dilution assay, C1-35 demonstrated a lower avidity for its target than the naturally occurring H.3-6 TIL. Alexander-Miller et al. proposed that high dose peptide stimulation could inhibit high avidity CTL while maximally stimulating low avidity CTL (35). Since g209-2M is characterized by high affinity for HLA-A*0201 (11), it is possible that the epitope density reached in vivo as a result of dose and route of administration led to extinction of high avidity CTL in this patient and stimulated the expansion of a low avidity clone, C1-35. This finding, if confirmed, will have important implications for the administration of vaccines, and studies of the avidity of the predominant CTL after vaccination could become necessary to find optimal dose ranges for peptide-based vaccines. It could be postulated that the vaccination led to the in vivo loss of highly reactive g209-specific CTL, and such loss could explain the progression of tumor after initial response. Furthermore, the repeated in vitro sensitizations with g209-2M could have skewed the original proportion of g209-specific CTL present in postvaccination PBMC. One cannot rule out that we may have missed other g209-specific CTL, possibly with higher avidity. To minimize this bias, we reduced the time in bulk culture to 14 days before cloning. Direct cloning from peripheral blood would be the preferred method with minimal in vitro bias. However, multiple attempts to directly clone PBMC resulted in poor efficiency and lack of generation of reactive CTL.

Although C1-35 demonstrated lower avidity for its target compared with a naturally occurring TIL, it could kill with high efficiency all HLA-A*0201-matched, gp100-expressing melanoma targets and could release IFN-γ in response to stimulation with several melanoma cell lines characterized by combined high expression of HLA-A2 and gp100. Gervois et al. have shown that the epitope density requirements necessary to stimulate target cell kill by CTL are 10- to 10,000-fold less than that required for induction of IL-2 and IFN-γ release by the same CTL (22). Tumor cells that could be efficiently lysed by MAA-specific CTL could not stimulate IFN-γ and/or IL-2 release unless exogenously supplemented with the appropriate peptide. In this study, we confirm this finding and we postulate that C1-35 could have been responsible for the initial response of the metastasis by killing melanoma cells, while the stimulatory requirements for its expansion and activation were provided by the systemic administration of g209-2M in IFA. At the time of tumor progression, although vaccine-induced stimulation was ongoing, most tumor cells became resistant to lysis by losing expression of target Ag. Comparison of FNA material obtained before and after vaccination revealed that the tumor had drastically decreased the fraction of cells expressing the gp100 over time. We hypothesize that this loss was due to specific killing of gp100-positive cells by CTL expanded by the vaccine, including C1-35. C1-35 appeared to still be present in the tumor mass at the time of progression (perhaps in response to few remaining gp100-expressing tumor cells or the effects of the still ongoing vaccination). However, its presence correlated with an inactive status, as none of 20 TIL bulk cultures expanded from the postvaccination FNA reacted to g209/g209-2M. This might indicate a dormant state of C1-35 in the tumor secondary to the decreased expression of gp100. The detection of TIL that recognize tumor cells, but not g209-2M or g209, suggests that gp100 loss by the tumor was counteracted, at that time point, by induction of CTL with another specificity, and underscores the dynamic and interactive nature of the immune response at the tumor site.

In this study, several observations could be made that would have escaped detection by monitoring the systemic immune response alone. First, CTL activated by the vaccine, although capable of recognizing and killing other gp100-expressing melanomas, were unable to recognize autologous tumor from a progressing metastatic lesion. The finding could be best explained by lost (or severely decreased) expression of the target MAA at that point in time. Second, despite the lack of target Ag by the tumor, C1-35 was found to localize at tumor site by molecular methods. The presence at tumor site, however, was associated with a dormant state, as this CTL could not be expanded by general proliferative stimuli consisting of IL-2 or OKT-3. Third, a new TIL emerged that could recognize an unidentified MAA in association with a restriction element unrelated to the vaccination (HLA-Cw*0702). Furthermore, the use of FNA allowed for serial sampling of the same metastasis without interference with its clinical course throughout treatment and afterward. This permitted a direct correlation between functional studies and therapeutic outcome.

This study illustrates the necessity of analyzing target tissue/host interactions at the site in which they are likely to occur. Such information may complement data obtained with the analysis of the systemic effects of vaccination and might enhance the understanding of the complex mechanisms underlying the success and failure of vaccination.

Acknowledgments

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A Model for CD8$^+$ CTL Tumor Immunosurveillance and Regulation of Tumor Escape by CD4 T Cells Through an Effect on Quality of CTL

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Understanding immune mechanisms influencing cancer regression, recurrence, and metastasis may be critical to developing effective immunotherapy. Using a tumor expressing HIV gp160 as a model viral tumor Ag, we found a growth-regression-recurrence pattern, and used this to investigate mechanisms of immunosurveillance. Regression was dependent on CD8 T cells, and recurrent tumors were resistant to CTL, had substantially reduced expression of epitope mRNA, but retained the gp160 gene, MHC, and processing apparatus. Increasing CTL numbers by advance priming with vaccinia virus expressing gp160 prevented only the initial tumor growth but not the later appearance of escape variants. Unexpectedly, CD4 cell depletion protected mice from tumor recurrence, whereas IL-4 knockout mice, deficient in Th2 cells, did not show this protection, and IFN-γ knockout mice were more susceptible. Purified CD8 T cells from CD4-depleted mice following tumor regression had more IFN-γ mRNA and lysed tumor cells without stimulation ex vivo, in contrast to CD4-intact mice. Thus, the quality as well as quantity of CD8$^+$ CTL determines the completeness of immunosurveillance and is controlled by CD4 T cells but not solely Th2 cytokines. This model of immunosurveillance may indicate ways to enhance the efficacy of surveillance and improve immunotherapy. *The Journal of Immunology*, 1999, 163: 184–193.

Improvement of T cell-mediated immunity for immunotherapy and development of vaccines has been one of the major strategies against cancer in this decade. Although many tumor-associated Ags were found in human cancer cells (1–10) and several trials have been conducted in a number of cancer patients (11–16), with a few exceptions (16), most of these therapies failed to induce an immune response sufficient to prevent further development of disease. The reasons for this poor success rate should be considered from the perspectives of both the factors in the tumor and those in the host. Lessons may be learned from the natural immunosurveillance against tumors expressing tumor Ags that could be applied to immunotherapy.

Despite the induction of a specific CTL response and appropriate help by helper T cells, it has been difficult to eradicate all of the tumor cells. There is accumulating evidence for escape mechanisms of tumor cells (17–19), including loss of Ag or class I expression, production of suppressive cytokines by tumor cells, and expression of Fas ligand on tumor cells (20–37). Considering the fact that escape variants expanded again after nearly complete rejection (20), it is important to determine how to prevent these tumor escape mechanisms to obtain durable remissions.

In this study, we examine whether CTL induced against a model viral tumor Ag can control the growth of tumor, how tumor cells escape from this immunosurveillance, and how we can prevent those escape variants. As a model tumor with a well-characterized Ag as a model viral tumor Ag, we used a BALB/c 3T3 fibroblast line transfected with HIV gp160 and with mutant ras and myc for tumorigenicity. Immunosurveillance of tumors expressing viral Ags may succeed, as is often the case for EBV-transformed B lymphocytes, or sometimes fail, as in the case of cervical carcinoma expressing human papillomavirus Ags, even in individuals who are not immunodeficient. We have previously characterized in depth the murine CTL response to an immunodominant determinant of gp160, called P18, contained within the V3 loop (38–48), that facilitates use of this Ag as a model viral tumor Ag.

There have been reports indicating that Th2-type cytokines down-regulated antitumor immunity (49, 50) and the activation of type 1 T cell responses produced antitumor immunity (51–54). In a cross-sectional epidemiological study of papillomavirus-related cervical neoplasia, we observed an inverse correlation between the fraction of individuals making a Th1 cytokine response and the degree of progression of disease (55). Thus, a shift to Th1-type cytokine production may be one goal for effective immunotherapy for tumors as well as virus infection. Therefore, we also address a question whether reduction of Th2-type cytokine production could enhance immunosurveillance. We found a novel striking enhancement of surveillance by CD4 cell depletion that cannot be explained primarily by elimination of Th2 cells.

Thus, we have taken advantage of the intriguing pattern of growth, spontaneous (immune-mediated) regression, and recurrence of this novel model tumor to investigate the relation between different cellular immune responses and tumor growth. We examined the role of CD8 and CD4 cells in the initial tumor regression and in preventing or facilitating tumor recurrence. We also examined the molecular mechanism of tumor escape from CTL in vivo. Unexpectedly, we discovered a novel important role for the quality of CTL, with respect to ex vivo activity and the amount of IFN-γ

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mRNA, in prevention of recurrence of tumor, and the regulation of this CTL quality by CD4 cells.

Materials and Methods

Mice and reagents

Female BALB/c mice were purchased from Charles River Breeding Laboratories (Frederick, MD). IL-4 knockout (KO)[2] and IFN-γ KO mice having the BALB/c background were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were kept under pathogen-free conditions and used at 6–10 wk old. Animal experiments were all approved by the National Cancer Institute (NCI) Animal Care and Use Committee.

Anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) for in vivo depletion were obtained from the Frederick Cancer Research and Development Center, NCI (Frederick, MD). FITC-conjugated anti-H-2D^d mAb (clone 34-2-12) was purchased from PharMingen (San Diego, CA). Mouse CD8 T cell subset enrichment columns were obtained from R&D Systems (Minneapolis, MN). Recombinant vaccinia vPE16, expressing the gp160 envelope protein of HIV-1 strain IIIB and control vaccinia vSC8, expressing β-galactosidase, were kindly contributed by Drs. Patricia Earl and Bernard Moss (National Institute of Allergy and Infectious Disease, Bethesda, MD) (56, 57). Trizol Reagent, Super Script cDNA synthesis reagents, and PCR SuperMixture were purchased from Life Technologies (Rockville, MD). NorthernMax, Stripe-EZ RNA Kit, and pT7 mouse IFN-γ probe template were obtained from Ambion (Austin, TX).

Tumor cells

15-12 are BALB/c 3T3 cells transfected with gp160 envelope protein of HIV-1 IIIB (38). 18Neo are BALB/c 3T3 cells transfected with the neomycin resistance gene alone as a control. 15-12RM was made from 15-12 by transfection with *Myc* and mutant *Ras* genes, containing the substitution of glycine to valine at position 12 of K-*ras* p21. All cells were maintained in T cell complete medium containing 0.2 mg/ml of geneticin (Sigma, St. Louis, MO).

CTL generation

Splenocytes from BALB/c mice previously immunized with 1×10^7 PFU of vPE16 were stimulated with irradiated BALB/c splenocytes pulsed with 1 μM peptide18 (P18) IIIB in a 24-well culture plate in complete T cell medium supplemented with 10% T-stim (Collaborative Biomedical Products, Bedford, MA). After 7 days of culture, viable cells were harvested and a CTL line against P18-IIIB was established by several restimulations with P18-IIIB-pulsed splenocytes. A CTL line for 15-12RM was derived from splenocytes of 15-12RM tumor-bearing mice taken on day 50 after inoculation of 15-12RM cells. These splenocytes were stimulated with 15-12RM cells, which were treated with mitomycin C (Sigma) (100 μg/ml for 45 min), plus irradiated splenocytes of normal BALB/c mice. The CTL line specific for 15-12RM was induced after several such restimulations. T cell complete medium is Biofluids (Rockville, MD) R2E Medium (a 50:50 mixture of RPMI 1640 and EHAA media) supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin, 5×10^{-5} M 2-ME, and 10% FCS.

CTL assay

Cytolytic activity against several target cells was assayed by a 4-h ^{51}Cr-release assay, as described elsewhere (58). Tumor cells harvested from tumor-bearing mice were used for target cells either on the same day when they were resected or after 1 wk of culture in complete T cell medium containing geneticin. Where indicated, CD8$^+$ T cells were purified from splenocytes using a mouse CD8$^+$ T cell subset enrichment column (R&D Systems) and used as effector cells. The percentage of specific ^{51}Cr release was calculated as: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton X-100. Spontaneous release was determined from target cells incubated without added effector cells.

Tumor inoculation

A total of 1×10^6 15-12RM cells in 200 μl of PBS were injected s.c. on the right flank of the mouse. Where indicated, 1×10^7 PFU of vPE16 or vSC8 were injected i.v. at 5 wk and 7 days before tumor inoculation. Some mice were treated i.p. with 0.2 ml of PBS containing either 0.5 mg of anti-CD4 mAb (clone GK1.5), anti-CD8 mAb (clone 2.43), or control rat IgG (ICN Pharmaceuticals, Costa Mesa, CA) starting 3 days before tumor cell injection and then twice a week.

Detection of mRNA by RT-PCR and Northern blot analysis

Total RNA was extracted from 5×10^6 tumor cells in Trizol reagent (Life Technologies). cDNAs were synthesized by extension of oligo(dT) primers using the Super Script Preamplification System (Life Technologies), according to the manufacturer's instructions. PCR of the cDNA was performed in a final volume of 50 μl containing each primer at 0.2 μM and Supermixture (Life Technologies) using the GeneAmp 9700 PCR system (Perkin-Elmer, Norwalk, CT). The amplification cycles were 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. After 25 cycles, PCR products were separated by 10% TBE gel electrophoresis and stained with Vista Green (Amersham, Arlington Heights, IL). The sequences of primers are as follows: hypoxanthine-guanine phosphoribosyl transferase (HPRT) (sense), 5′-GTTGGATACAGGCCAGACTTTGTTG-3′; HPRT (antisense), 3′-GAGGGTAGGCTGGCCTATGGCT-5′; gp160 v3 loop (sense), 5′-GCT GTTAAATGGCAGTCTAGC-3′; gp160 v3 loop (antisense), 3′-CGT TAGGAGTCCTCCCCTGGG-5′. Northern blot analysis was performed using NorthernMax and Strip-EZ RNA Kit (Ambion), according to the manufacturer's instruction. Total cellular RNA was used, and the integrity of RNA was tested by electrophoresis in 2% agarose gel. A total of 15 μg of RNA was loaded per lane and transferred onto nylon membranes. After prehybridization, the ^{32}P-labeled probe for IFN-γ was hybridized at 65°C overnight. Then the membrane was washed with low- and high-stringency solution. Autoradiography was conducted at −70°C for up to 4 days by using Kodak (Rochester, NY) BioMax-MS film. The same membrane was stripped and used to rehybridize with a GAPDH probe as an internal control.

Flow cytometry

18Neo and tumor cells were stained with FITC-conjugated anti-H-2D^d mAb to detect the expression of class I molecules. They were analyzed by FACScan using CellQuest software (both from Becton Dickinson, Mountain View, CA).

Histological examination

Tumor tissues were excised on the indicated days after inoculation of tumor cells, fixed in 10% v/v formalin solution, and processed for paraffin embedding. Sections were cut according to standard procedures and stained with hematoxylin and eosin.

Results

15-12RM tumor regressed after initial growth and recurred in vivo

15-12RM cells were injected s.c. on the right flank at day 0. Tumors initially started growing within 5 days after inoculation, and they reached about 8–10 mm diameter at approximately day 7. The tumors then began to regress spontaneously and disappeared after ~10–12 days. The growth rate decreased at this time, even in the occasional small tumors that remained. However, the mice in which tumors had regressed initially, even beyond the point of detection, developed tumors again between 20 and 30 days after inoculation. These did not regress in this second growing stage (Fig. 1, *a* and *b*).

Vaccinia viruses were tested to induce tumor-protective immune responses in vivo. Vaccinia virus vPE16, which expresses gp160 in infected cells (56), was injected i.v. twice with a 30-day interval, and tumor was inoculated 7 days after the second immunization. Though 15-12RM tumor cells injected into control vaccinia vSC8-immunized mice did not always behave identically to normal mice, they shared a similar pattern of development of tumors, including the three phases of initial growth, regression, and regrowth. In contrast to normal and vSC8-immunized mice, mice immunized with vaccinia virus vPE16 showed a different process for growth of tumors. They did not manifest the primary development of tumors, but the tumors appeared only late, after day 30, and behaved like the tumors seen in the recurrence phase of normal and vSC8-immunized mice, without first undergoing regression (Fig. 1*c*). These

[2] Abbreviations used in this paper: KO, knockout; HPRT, hypoxanthine-guanine phosphoribosyl transferase.

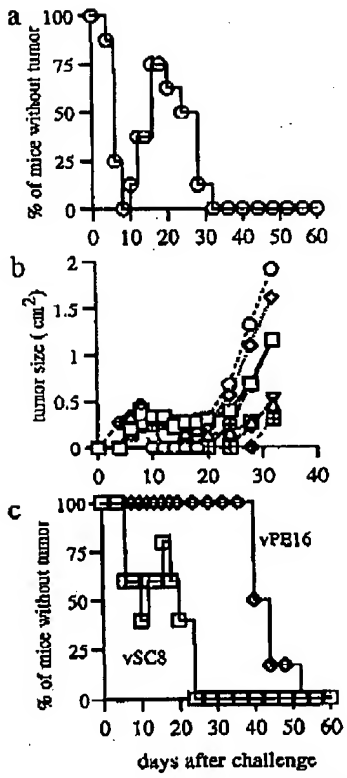

FIGURE 1. The appearance and regression of tumors in normal mice and vaccinia-immunized mice. *a*, A total of 1×10^6 15-12RM cells were injected s.c. on the right flank of wild-type BALB/c mice. The size of tumor was measured every 2 days until day 20 after inoculation and later every 4 days. Tumors having more than a 4-mm diameter were defined as positive. *b*, The kinetics of the size of tumors (product of perpendicular diameters) of eight mice. *c*, Mice were immunized twice (1 mo apart) i.v. with 5×10^6 PFU/200 μl of vPE16 or vSC8. Then, 7 days after the second injection of vaccinia virus, 1×10^6 15-12RM cells were inoculated in the same way as in *a*. In all three panels, eight mice were used per group. Similar results were found in three independent experiments.

vPE16-immune mice also had $CD8^+$ CTL specific for HIV-1 gp160 peptide P18-IIIB (data not shown, and Refs. 38 and 48). Thus, immunization against gp160, inducing specific CTL, could protect mice from the initial growth of tumors, but not from the later development observed 30 days after inoculation of tumor cells.

Regression depends on the existence of CD8 T cells

From the above results, we hypothesized that $CD8^+$ CTL might play a role in the initial regression of tumor. To investigate the role of CD4 and CD8 T cells in the regression and growth of tumor, nonimmunized mice were treated with Abs against CD4 or CD8 molecules. Flow cytometric analysis showed that >98% of CD4 and CD8 T cells were depleted by the treatment with the respective Abs (data not shown). In the anti-CD8 Ab-treated groups, tumors grew initially in the same time period as in control mice, but they continued growing without regression (Fig. 2*a*). Their growth rate did not decrease at 10–12 days, when the tumors of control mice regressed. Although anti-CD4 Ab treatment did not result in any significant change in the process of growth and regression of tumors up to day 30 after the challenge with tumor cells, depletion

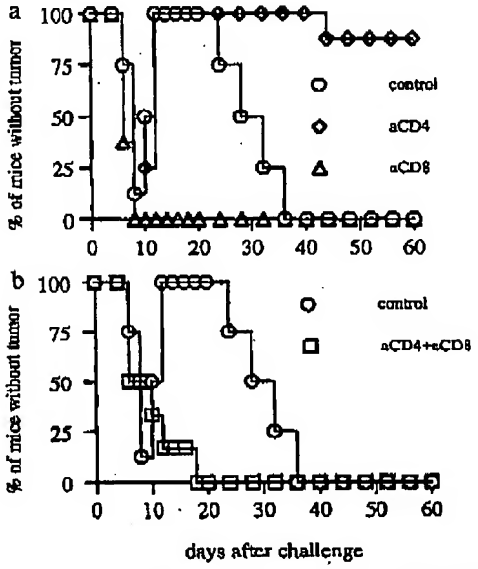

FIGURE 2. The development of tumors in CD4- or CD8-depleted mice. A total of 0.5 mg of anti-CD4 (GK1.5), anti-CD8 (2.43), or both Abs was injected i.p. on three consecutive days before inoculation of 1×10^6 15-12RM cells (eight mice/group). Abs were given twice a week after inoculation of tumor cells. Tumors having more than 4 mm of diameter were defined as positive. Comparable results were obtained in three independent experiments.

of CD4 cells unexpectedly protected the mice from later regrowth of tumor (Fig. 2*a*). When CD8 cells were depleted simultaneously with depletion of CD4 cells, the tumors continued to grow without regression, as in the anti-CD8 Ab-injected group (Fig. 2*b*). Thus, CD8 cells were necessary for initial rejection of the tumor, and the depletion of CD4 cells somehow prevented the regrowth of tumors after regression. When anti-CD8 Ab were given to the CD4-depleted group only after regression (starting from day 21), tumors developed in only some of the mice, despite the fact that there were no CD8 cells (data not shown). This observation is consistent with the interpretation that, in anti-CD4 treated mice, 15-12RM tumor cells did not survive after the initial regression, so the absence of CD8 cells at the later time point was no longer relevant.

Histological examination confirmed infiltrating lymphocytes

Since it was suggested that the growth and regression of tumors were regulated by immunosurveillance, especially CD8 T cells, we performed a histological examination of tumors to see whether they were compatible with the observation described above. Specimens were harvested from 15-12 RM tumor cells injected into normal mice at different time points after inoculation. At day 9, when initial tumors were growing, focal sparse mixed cellular (chronic) inflammatory infiltrates were noted within the tumor mass. At day 13, when growth stopped, the number of infiltrating lymphocytes seen around tumor cells increased (Fig. 3), and, when there were no tumors on the surface of the flank at day 19, the lymphocytic response reached a maximal level (Fig. 3). These findings raise the possibility that these infiltrated lymphocytes contributed to the regression of tumors. In contrast, when tumor reappeared and grew again after regression, the recurrent tumors had a more spindle-shaped morphology and were relatively devoid of infiltrating lymphocytes at days 33 and 46 (Fig. 3). Thus, the histology was consistent with a lack of a cellular immune response to the recurrent tumors.

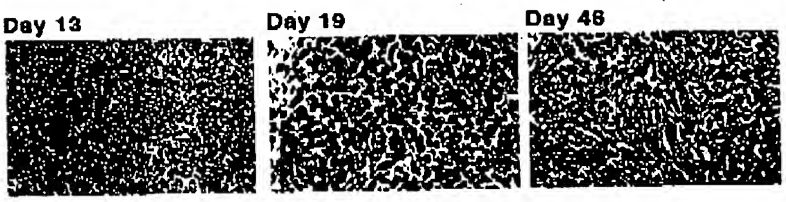

FIGURE 3. Histological findings of tumor sections on several days after inoculation of 15-12RM tumor cells. At day 13, initial tumor was observed on the right flank of the mice. Tumor regressed and it was not seen with the naked eye on day 19. After regression, tumor appeared and grew again (day 46). Hematoxylin and eosin staining, magnified ×225 (day 13), ×500 (day 19), and ×225 (day 46).

Recurrent tumors developing after regression were resistant to 15-12RM-specific CTL

CTL specific for 15-12 RM tumor could also lyse target cells presenting P18-IIIB from the v3 loop of the HIV-1 envelope (Fig. 4*b*). Likewise, P18-IIIB-specific CTL, which were induced from a vPE16-immunized mouse by stimulation with 1 μM P18-IIIB-pulsed spleen cells, could recognize 15-12 RM tumor cells (Fig. 4*a*). Therefore, we utilized CTL against both 15-12 RM tumor cells and P18-IIIB to determine the susceptibility of the tumor cells to cytotoxicity. Our objective was to examine how tumors recurred after initial regression, despite the rejection mediated by CD8 T cells or the immune protection against initial growth observed in vPE16-immunized mice. The possible explanations of this observation could be either alteration of tumor cells (escape variants) or induction of tolerance in responding T cells. To examine the former possibility, tumor cells were recovered from mice at different stages in vivo and used as target cells in the CTL assay. While the tumor cells that grew initially retained sensitivity for 15-12 RM CTL, tumor cells from the recurrent stage could not be lysed by the same CTL (Fig. 5, *a* and *b*). Both freshly isolated cells and cultured cells, which were selected by growth in culture medium containing geneticin (to kill cells that had lost the Neo^R gene), showed the same results. As mentioned above, vPE16-immunized mice developed tumors late without an early growth and regression phase. Tumor cells from these animals were not killed by 15-12 RM CTL (Fig. 5*a*). However, tumor cells from normal or vSC8-immunized mice treated with anti-CD8 mAb were killed by 15-12 RM CTL (Fig. 5*c*). Moreover, 15-12RM-specific CTL could kill tumor cells harvested from vPE16 immunized, anti-CD4 plus anti-CD8 mAb-treated mice (data not shown).

To test the possibility that contamination of CTL-resistant tumor cells in the cultured 15-12RM clone was the origin of recurrent tumor in vivo, 15-12RM cells were subjected to killing once or twice in vitro by P18 CTL, and then surviving cells were used as target cells for the CTL assay after several days of culture. We hypothesized that, if there are very few resistant tumor cells in the tumor cell suspension used for injection, these preexisting resistant tumor cells should gradually become the majority of target cells after selection by killing of sensitive cells by CTL in vitro. As shown in Fig. 6, 15-12RM were lysed by P18 CTL, even after two rounds of selection, in three of four experiments. Moreover, in the one case when cells became resistant after killing by CTL, although they were lysed after first round killing at the same level as original 15-12RM, they showed <5% of lysis after the second round. Therefore, the data suggest that it is unlikely that preexisting resistant tumor cells grew after regression of susceptible tumor cells in vivo. In summary, 15-12 RM cells before the tumor regression caused by CD8 T cells were susceptible to lysis by 15-12 RM CTL, but they did not maintain this character when they recurred after initial regression. Although vPE16-immunized mice showed early protection against tumor growth instead of growth and regression of tumor, 15-12 RM tumors that grew out in these mice after day 30 were resistant to gp160-specific CTL.

Detection of mRNA encoding the gp160 v3 loop in resistant and nonresistant tumors

To explore the mechanism of tumor resistance to CTL lysis, we examined the expression of the class I molecule $H\text{-}2D^d$ on the resistant tumor cells because P18-IIIB requires D^d to be presented

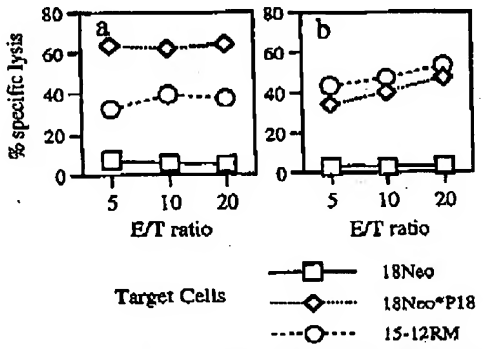

FIGURE 4. CTL lines established after several stimulations with spleen cells pulsed with 1 μM of P18-IIIB peptide (*a*) or with 15-12RM tumor cells (*b*) can lyse the same target cells. Target cells used were 18Neo (□), 18Neo pulsed with 1 μM of P18-IIIB (◇), and 15-12RM cells (○).

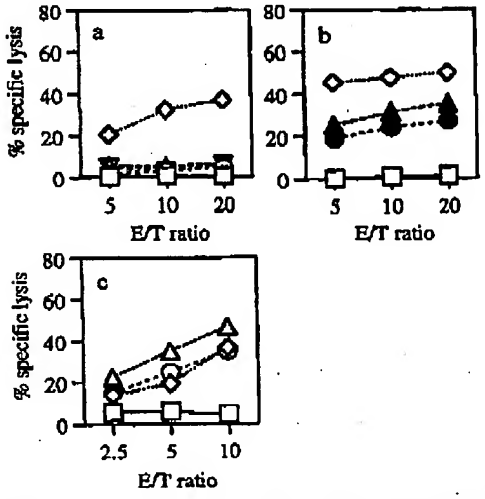

FIGURE 5. Sensitivity to CTL lysis of tumor cells recovered from BALB/c mice treated in various ways. In all panels, 18Neo (□) and 15-12RM cells (◇) were used as target cells in a CTL assay with the 15-12RM-specific CTL line as effector cells. *a*, Tumor cells harvested from naive mice at recurrent stage (○ and △), harvested from vSC8-immunized mice at the same stage (×) and from vPE16-immunized mouse (▽) were used as target cells. *b*, Tumor cells recovered during initial growth of tumors from naive mice were lysed by 15-12RM CTL (● and ▲). *c*, Tumor cells from anti-CD8-treated mice were lysed by the CTL line specific for 15-12RM (○ and △).

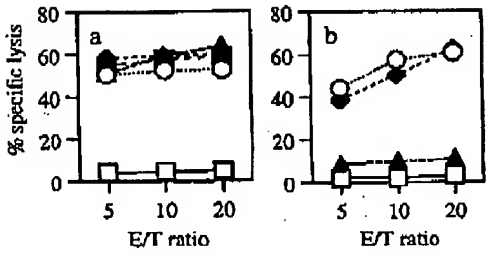

FIGURE 6. Sensitivity to CTL lysis of surviving tumor cells. *a*, After killing twice by P18-IIIB CTL in vitro, 15-12RM tumor cells were cultured for several days in G418 containing complete T cell medium, and expanded cells were tested as target cells in a CTL assay with P18-IIIB CTL. 15-12RM cells after two rounds of selection from three different experiments were shown as closed symbols in *a*. *b*, Surviving cells after the first round (◆) and second round (▲) of killing by P18-IIIB CTL from a fourth experiment were cultured in G418 containing complete T cell medium, and expanded 15-12RM cells were used as target cells in a CTL assay with P18-IIIB CTL. Open squares and open circles are 18 Neo, and original 15-12RM tumor cells used as target cells, respectively.

to T cells. As shown in Fig. 7*a*, resistant tumor cells harvested from mice expressed D^d at the same level as the original 15-12RM cells. Also, resistant tumor cells could be lysed by P18 CTL when they were pulsed with P18-IIIB or infected with vPE16 (Fig. 7*b*). These results showed that class I expression on the resistant tumor cells is intact and that resistant tumor cells can process and present the endogenously expressed Ag normally. Therefore, we asked if they had lost expression of the Ag. It was impossible to detect the

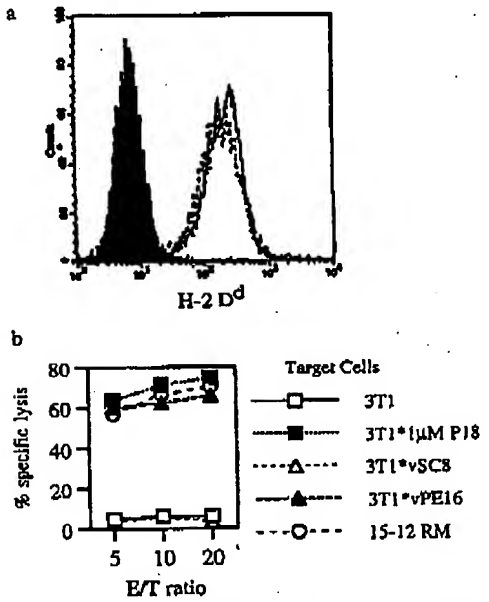

FIGURE 7. Resistance is not due to loss of MHC expression or Ag processing and presenting function. *a*, FACS analysis of H-2D^d expression on the tumor cells. The original 15-12RM cells (----) and tumor cells harvested at the regrowth stage (——) were stained with FITC-conjugated anti-H-2D^d mAb. The shaded area under the curve shows control staining with FITC-conjugated mouse IgG2a (PharMingen). *b*, CTL assay with P18-IIIB CTL for effector cells when resistant 15-12RM tumor cells (3T1) were pulsed with P18-IIIB (■) or infected with vSC8 (△), vPE16 (▲) as target cells. □ and ○, Resistant 15-12RM tumor cells and original 15-12RM tumor cells used as target cells, respectively.

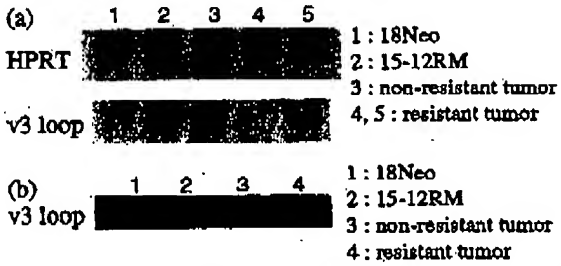

FIGURE 8. mRNA expression in resistant and nonresistant 15-12RM tumor cells detected by RT-PCR (*a*). The primers used were for v3 loop of gp160 and for HPRT as internal control. *b*, DNA extracted from various 15-12RM cells was amplified with primers for v3 loop. Similar results were obtained in three independent experiments.

expression of gp160 on the 15-12RM cells by Western blot analysis and flow cytometry. Thus, we compared the expression of mRNA in resistant and nonresistant tumor cells by RT-PCR. Fig. 8*a* shows that mRNA for the gp160 v3 loop was absent in the resistant tumor cells, while nonresistant tumor cells and the original 15-12RM cells retained clear message. Next, we examined if this mRNA defect resulted from the loss of the transfected gene or a mutation at the either of the primer regions of v3 loop. DNA was isolated from 5×10^6 tumor cells and amplified by PCR. There were the clear bands of amplified DNA by v3 loop primers for the resistant tumors, as well as nonresistant tumors (Fig. 8*b*). These PCR products were cloned and then sequenced from three resistant tumors and two nonresistant tumors harvested from mice and the original 15-12 RM cells. There was neither deletion of genes nor point mutations in the v3 loop region (data not shown). These results suggested that the tumors developed resistance against CTLs for P18-IIIB by decreasing the expression of mRNA encoding the gp160 v3 loop.

Loss of tumor-specific CTL does not account for the tumor regrowth

As mentioned earlier, the regrowth of tumor could be due either to the development of escape variants of the tumor, or to loss of CTL activity when the tumors reappear. Although the evidence above pointed to the outgrowth of resistant variants of the tumor, we also wanted to examine the possibility of CTL loss. Mice with large recurrent tumors were examined on day 62 after tumor inoculation. Spleen cells were restimulated for 6 days with the original tumor cells 15-12RM as stimulators, and then tested in a lytic assay on both 15-12RM targets and on 18Neo BALB/c 3T3 fibroblasts either with no peptide as a negative control or pulsed with 1 μM peptide P18-IIIB (Fig. 9). As can be seen from the two mice shown, a very vigorous CTL response was still present, specific for both HIV-1 gp160 peptide and tumor cells, even after substantial outgrowth of recurrent tumor. Thus, tolerance induction cannot account for the observed outgrowth of tumor.

These results, combined with the observation mentioned earlier that mice treated with anti-CD4 from the start but then treated with anti-CD8 only from day 21, after tumor had regressed, still mostly did not grow recurrent tumor, suggest that the protective effect of anti-CD4 is to allow the complete regression of tumor, so that no tumor is left to regrow once CD8 cells are eliminated. In the CD4-intact mice, the tumor regression is incomplete, and the tumor recurs despite the continued presence of CTL. Thus, we wondered whether there was some qualitative functional difference in the CTL that can complete the elimination of tumor in the absence of CD4 cells but not in their presence.

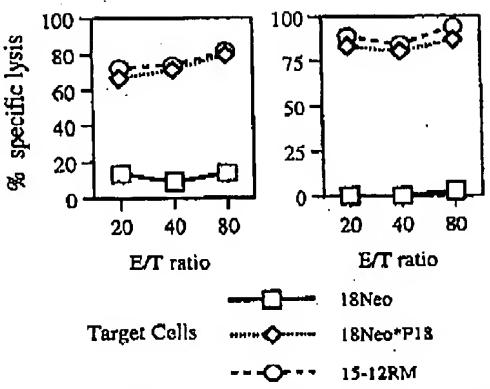

FIGURE 9. Mice retain high levels of tumor-specific CTL even during the phase of tumor recurrence. The splenocytes were recovered from the mice with recurrent tumor at day 62 after injection of 10^6 15-12RM tumor cells. In 24-well plates, 4×10^6 splenocytes were restimulated with 10^5 15-12RM cells and 2×10^6 normal splenocytes. The cultured cells were used for CTL assay at day 6 of culture at the E:T ratios shown. Targets were either 15-12RM cells, or 18Neo fibroblasts either without peptide or pulsed with 1 μM P18-IIIB peptide, as indicated. The two panels are representative data of two individual mice. Similar results were obtained if the splenocytes were restimulated with 1 μM P18-IIIB peptide instead of tumor cells (data not shown).

Production of IFN-γ by CD8 cells contributes to protection against the regrowth of tumors in CD4-depleted mice

As described above, depletion of CD4 cells, including Th cells, led to the protection against regrowth of 15-12RM tumors after regression. Since previous literature suggested that Th1 cells played an important role in protection against viral infection and tumor development (53, 54, 59), we utilized IFN-γ KO mice and IL-4 KO mice as models of Th1/Th2 imbalance to examine the influence of these cytokines on tumor growth and recurrence and on the prevention of regrowth by anti-CD4 mAb injection. Though some of the IFN-γ KO mice rejected tumors after initial growth, tumors regrew within 20 days after the inoculation, statistically significantly more rapidly than the control wild-type mice (Fig. 10*b*) ($p < 0.005$, wild type vs IFN-γ KO, Log-Rank test). On the other hand, surprisingly, IL-4 KO mice did not significantly differ from control wild-type mice with regard to the pattern of tumor development. They were not protected against regrowth of tumors as observed with anti-CD4 treatment, even though they had a skewed balance toward Th1 caused by the absence of IL-4 (Fig. 10*c* compared with Fig. 10*a*). When CD4 cells were depleted before tumor inoculation, all the mice showed better protection than mice injected with control rat IgG Ab (ICN Pharmaceuticals) in each group (Fig. 10, *a–c*). The results using IL-4 KO mice indicate that the complete loss of IL-4 and the great diminution of other Th2 cytokines that are in part dependent on IL-4 for their production (66–69), does not mimic the effect of CD4 depletion and, therefore, does not explain the striking protection in CD4-depleted mice. However, since tumors could regrow in CD4-depleted IFN-γ KO mice (Fig. 10*b*), these results suggested that IFN-γ produced by CD8 cells or NK cells played an important role in protection against regrowth of tumors in CD4-depleted mice.

To investigate further the mechanism of the protection produced by CD4 depletion, we purified CD8 T cells from splenocytes of untreated and CD4-depleted mice on day 14 after the inoculation of 15-12RM cells to compare the CTL activity. Tumors grown on the right flank initially disappeared at this time point in all the mice in both groups. As shown in Fig. 11*a*, $CD8^+$ T cells from CD4-

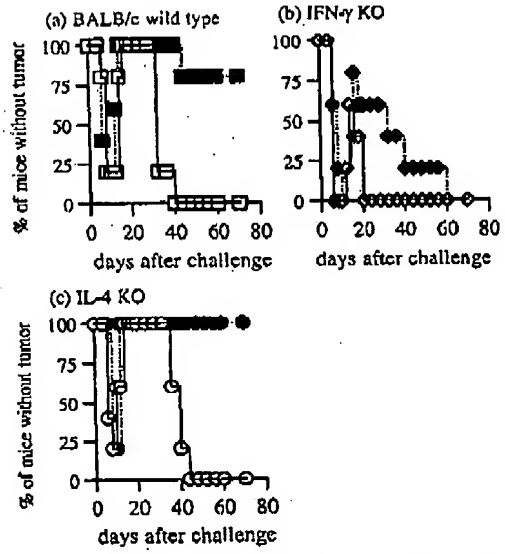

FIGURE 10. The development of tumors in wild-type (*a*), IFN-γ KO (*b*), and IL-4 KO (*c*) BALB/c mice (five mice/group). Closed and open symbols in all panels indicate CD4-depleted group and control rat IgG injected group, respectively. Tumors were observed and measured in the same way as Fig. 1 (*a*). Comparable results were found in three independent experiments.

depleted mice, without restimulation in vitro, could kill 15-12RM target cells, as well as 18Neo pulsed with 1 μM of P18-IIIB, while $CD8^+$ cells from tumor-inoculated, normal mice could not kill

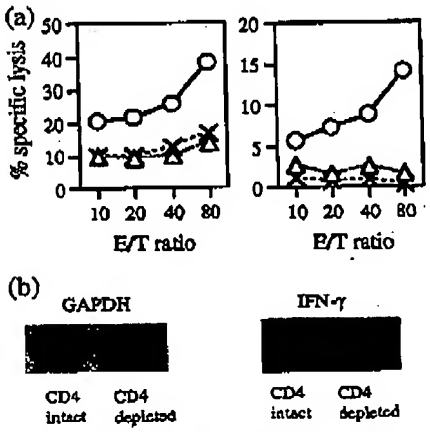

FIGURE 11. *a*, CD8 T cells purified from splenocytes of CD4-depleted, 15-12RM-inoculated mice (○) had greater CTL activity for both 18Neo pulsed with 1 μM of P18-IIIB (*left panel*) and 15-12RM cells (*right panel*) without any in vitro restimulation than those from 15-12RM-injected, normal mice (△ and ×). *b*, Northern blot analysis for IFN-γ and GAPDH mRNA. Spleens were harvested at day 14 after inoculation of tumor cells. Total RNA was extracted from CD8 T cells purified from splenocytes of CD4-depleted, 15-12RM-inoculated mice and splenocytes of 15-12RM-injected, untreated mice. Northern blot analysis was performed as described in *Materials and Methods*. Densitometry showed IFN-γ mRNA expression in relation to GAPDH mRNA expression from CD8 T cells in tumor-inoculated, CD4-depleted mice is four times as much as in tumor-inoculated CD4-intact mice. Similar results were obtained in three different experiments.

15-12RM cells without restimulation. Also, purified CD8 T cells from CD4-depleted mice injected with tumor could lyse 18Nco pulsed with 1 μM of P18-IIIB better than those from normal mice injected with tumor (about 15% vs 40% specific lysis at 80:1 E:T ratio). To further explore the functional difference between these CTL, we examined the expression of IFN-γ mRNA in these $CD8^+$ T cells. Consistent with the results of the CTL assay, we could detect four times as much IFN-γ mRNA from CD8 T cells in CD4-depleted mice as from $CD8^+$ cells in tumor-inoculated untreated mice (Fig. 11*b*). These results indicated that the quality of CD8 T cells in CD4-depleted mice was different from that in normal mice with regard to IFN-γ mRNA expression, as well as CTL activity without restimulation, properties that may be important for the protection against recurrence of tumor.

Discussion

In this study, we present a novel manipulable animal model of tumor immunosurveillance in which we can evaluate escape mechanisms of tumor cells from immunosurveillance, the role of $CD8^+$ T cells in causing regression and preventing local recurrence, and the adverse role of $CD4^+$ T cells in local recurrence. As a model of soft tissue tumor in vivo, we utilized the transfectant fibroblast cell line expressing myc and the K-ras point mutant along with HIV-1 gp160 as a tumor Ag. Although not a natural tumor Ag, this viral Ag is not unlike other viral Ags expressed in certain human tumors, such as human papillomavirus Ags in cervical carcinoma. This tumor has the histological appearance of and invades muscle tissue like a soft-tissue sarcoma. It may serve as a model of human tumors that express viral Ags, such as cervical carcinoma induced by human papillomavirus or lymphoid malignancies induced by HTLV-I. CTL induced against this tumor cell line, 15-12RM, could recognize target cells pulsed with an immunodominant CTL epitope peptide P18-IIIB of the gp160 v3 loop (38), as well as tumor cells themselves. Prevention of regression by $CD8^+$ cell depletion in vivo indicated that the regression was CD8-dependent. However, in unmanipulated mice, the tumors virtually always recurred, despite the continued presence of CTL (Figs. 1 and 9). Thus, this system serves as a model of tumor immunosurveillance. In the clinical situation in humans, many tumors express potential tumor Ags, and many subclinical tumors may undergo this type of spontaneous regression, but only the ones that escape immunosurveillance and reach the recurrent stage, as in our model, are detected clinically. If we could understand what prevents immunosurveillance from achieving complete regression and allows tumor recurrence and later metastasis, we might be able to overcome this obstacle and prevent tumor recurrence. This understanding is also critical to designing optimal immunotherapy of cancer.

A potentially important clue to this problem came from the surprising effect of CD4 depletion. Depletion of CD4 T cells did not change the pattern of initial tumor growth and regression, but it inhibited recurrence of the tumor. How could one explain the prevention of recurrence of the tumor in CD4-depleted mice? In accordance with the fact that CTL induction is associated with a Th-1 type immune response, it has been shown that CD4 T cells that can secrete Th1-type cytokines have a beneficial role in protection against tumor development (53, 54). Likewise, we previously reported that IL-2 production by human peripheral lymphocytes in response to human papillomavirus Ags is inversely associated with disease status (55). McAdam et al. (60) showed that murine carcinoma cells transfected with IL-2 and IFN-γ were more likely to be rejected than parental cells when implanted in BALB/c mice. On the other hand, the shift from Th1-type to Th2-type cytokine production was found in progressive cancer patients (61, 62), and T cells harvested from tumor-bearing hosts produced only Th2-type cytokines when they were stimulated in vitro (63). In addition to these findings, Th2-type cytokines could even accelerate the experimental pulmonary metastasis of melanoma (49).

Therefore, we hypothesized that the shift to a Th2-type response to gp160 could be the cause of failure of complete tumor regression, allowing recurrence of tumors after regression. We used IFN-γ KO mice and IL-4 KO mice as a model of Th1/Th2 imbalance (64) to address this question. Although the IFN-γ KO mice could suppress the initial growth of tumor, these tumors finally developed earlier than those in control BALB/c mice, suggesting a role for IFN-γ. However, other data suggested a critical role for CD8 T cells: 1) vPE16-immunized mice that have P18-IIIB-specific CTL before inoculation with tumor did not have initial growth of tumors; 2) CD8-depleted mice could not inhibit tumor growth at all; 3) Recurrent tumors had all become resistant to lysis by CTL; and 4) We could induce a CTL response even from 15-12RM tumor-bearing IFN-γ KO mice by stimulation with P18-IIIB in vitro (data not shown). Thus, we concluded that both lytic activity by CD8 T cells and production of IFN-γ are necessary for the regression of the initial tumor in our model system.

Prior immunization with vaccinia virus vPE16, which induces gp160-specific CTL, could prevent the initial growth of tumor completely, but not its recurrence later. This result indicated that the increase of CTL precursors for P18-IIIB contributed to clearance of tumor cells, but could not eradicate them. In contrast, in CD4-depleted mice, initial growth and regression of tumors were still observed but the subsequent recurrence was prevented. Thus, the increase in CD8 T cell numbers alone cannot explain the benefit of CD4 depletion. When CD8 T cells were depleted by the injection of anti-CD8 mAb starting from day 21 after inoculation of tumor cells in mice treated with anti-CD4 mAb from the beginning, the tumors developed in only 25% of these CD4-depleted mice (data not shown). This result indicated that CD4-depleted mice might eliminate all of the tumor cells before the critical point for recurrence, so that once the tumor cells were gone, the $CD8^+$ cells were no longer necessary. In contrast, in the presence of CD4 cells, the clearance of tumor by CD8 cells was incomplete, even though CTL remained present during tumor recurrence (Fig. 9).

However, unexpectedly, IL-4 KO mice did not mimic CD4-depleted mice in that they could not stop the development of tumor after regression even though they had a shift to Th1-type response in general (Fig. 10) (67, 65). IL-4 is necessary for the normal development of Th2 responses and production of other Th2 cytokines (66–68), although some production of IL-5 and IL-10 can still occur (69). The IL-4 KO mice thus indicate that IL-4 is not required for the CD4-mediated prevention of complete regression, and probably other Th2 cytokines, which are greatly diminished in the IL-4 KO mice, also do not account completely for the inhibitory effect of CD4 cells on the elimination of tumor. Therefore, although a Th1-type response could contribute to the rejection of initial tumors and a shift to a Th2-type response could interfere with this protection, even a substantial skewing toward Th1-type response by elimination of IL-4 was not sufficient to prevent recurrence of tumors after regression. CD4 depletion must accomplish more than just Th2 depletion.

Koeppen et al. (70) observed that anti-CD4 treatment of mice increased the frequency of rejection of an allogeneic tumor expressing a foreign class I MHC molecule. Rakhmilevich and North (71) showed that elimination of CD4 T cells augments the antitumor effect of IL-2 therapy in mice bearing an advanced sarcoma by releasing CD8 T cell-mediated immunity from T cell-mediated suppression. Martinotti et al. (72) reported that tumor infiltration by the CD8 T cells was inhibited by CD4 T cells, but the tumors

were unusual in being transduced with the gene for IL-12. In that report, they postulated that CD4-mediated suppression is exerted on CD8 expansion and on the ability of CD8 T cells to infiltrate tumor nodules. However, we could observe infiltration of lymphocytes in the tumor tissue not only in CD4-depleted mice but also in untreated mice. Also, we found several lines of evidence for a qualitative difference in CD8 cells from CD4-depleted mice that appeared to contribute to more effective tumor elimination. First, CD8 T cells purified from splenocytes of CD4-depleted and tumor-injected mice had higher CTL activity specific for 15-12RM cells than CD8 T cells from tumor-inoculated CD4-intact mice, even without any stimulation in vitro. This result indicated that CD8 T cells from CD4-depleted mice were already activated to kill tumor cells efficiently in vivo. Second, this better lytic activity of CTL was correlated with the expression of IFN-γ mRNA. Thus, $CD8^+$ T cells from CD4-depleted mice, which could secrete more IFN-γ, could play an important role in prevention of recurrence of tumor in our model system. We conclude that a qualitative alteration of CD8 T cells following depletion of CD4 T cells could account for protection from recurrence of tumor, whereas the reduction in Th2-type cytokines in IL-4 KO mice was not sufficient.

We previously reported the importance of the quality of CTL as well as the quantity of CTL for viral clearance, in a study in which high-avidity CTL specific for P18-IIIB could protect better against virus challenge than low-avidity CTL (48). We are now investigating whether the quality of CTL has any correlation with high- or low-avidity CTL and whether there is any relation between CD4 depletion and the appearance of escape variant tumor cells.

Even during the recurrence phase of new tumor growth, a CTL response was detected in spleen cells from these tumor-bearing mice after the stimulation with P18-IIIB-pulsed spleen cells or 15-12RM cells (Fig. 9). This observation indicated that the exhaustion or tolerization of responding cells against P18-IIIB was not the cause of tumor recurrence after regression. There are several possible remaining explanations for this escape mechanism of tumor cells from immunological destruction (19). Besides the host factors that can suppress immune defenses against tumor cells already discussed, there were several reports of Ag loss of tumor variants (17, 18, 20). Selection of Ag loss, epitope loss, or class I loss variants might lead to recurrence of tumor. In this study, recurrent tumor cells became resistant (resistant tumor cells) to CTL that could lyse both the original tumor cells and also the tumor cells recovered from the initial growth stage before regression (nonresistant tumor cells). The resistant tumor cells remained class I-positive at the same level as the original tumor cells by FACScan, and could process and present endogenous Ag, gp160, as shown by their ability to be lysed by P18-IIIB-specific CTL when infected with recombinant vaccinia virus expressing gp160. Since gp160 on 15-12RM cells could not be detected by Western blot and flow cytometric analyses, we investigated the presence of DNA and the expression of mRNA. We could detect amplified DNA of the v3 loop (containing P18-IIIB) of gp160 from resistant tumors as well as from the original 15-12RM and nonresistant tumors, and the DNA did not contain any point mutation at any coding position within or near the v3 loop. However, only resistant tumor cells lost the expression of mRNA for the v3 loop. Moreover, tumor cells recovered from CD8-depleted mice could be lysed by CTL specific for P18-IIIB. Thus, this acquisition of resistance against CTL occurred only in the presence of immune pressure by CD8 T cells. These resistant tumor cells could grow in normal BALB/c mice without regression and were not killed by CTL after several weeks of culture in G418-containing medium, which means that escape variants selected by CD8 T cells were stable both in vivo and in vitro, in contrast to the situation in another report (73). Our results make contamination of escape variants in the original 15-12RM cells unlikely. In vivo, the tumor is removed from the G418-containing selection medium used to select for neomycin-resistant cells. However, this removal is unlikely to play a role in the escape for several reasons. First, the original 15-12 cells were made by cotransfection with Neo^R and *gp160* genes on different plasmids, so that Neo^R could be used to select for cells that took up DNA in the original transfection, but selective pressure from G418 should not affect retention of the gp160 gene (38). Second, the original 15-12 transfectants continue to express gp160 for at least 3 mo in culture without G418. Third, the failure to develop resistant tumors in the CD8-depleted mice (in the absence of G418), as just mentioned, implies that the selection for resistance requires CD8-mediated immune pressure. Fourth, the gene for gp160 is retained, but just the mRNA expression is lost. Thus, it is very important to know how CTL pressure can cause the decrease of mRNA expression of the epitope region in tumor cells to allow escape from immunological surveillance.

In conclusion, this model has allowed us to begin to dissect some of the mechanisms mediating and regulating tumor immunosurveillance. $CD8^+$ CTL appear to be critical for causing tumor regression, but quantity of CTL alone is not sufficient. Rather, qualitatively different CTL that produce more IFN-γ and remain activated in vivo may be critical. The qualitative difference in CTL is influenced by $CD4^+$ cells that regulate the $CD8^+$ response, but this regulation cannot be explained simply by the Th1/Th2 balance. Further studies to determine the mechanism of this regulation will be important for designing optimal immunotherapy. It may be valuable to induce CTL producing high amounts of IFN-γ, as well as having high avidity, to obtain good quality CTL for immunotherapy. Such CTL could be a useful component of a strategy to prevent escape variants of tumor cells and to prevent recurrence of tumors to control malignant disease. Concurrent abrogation of the inhibitory effects of CD4 cells without eliminating IFN-γ production may provide a successful concerted approach to cancer immunotherapy.

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Identification of Multiple Antigens Recognized by Tumor-Infiltrating Lymphocytes From a Single Patient: Tumor Escape by Antigen Loss and Loss of MHC Expression

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Abstract: The authors describe a patient who experienced recurrence of metastatic melanoma after an initial dramatic response to immunotherapy using peptides derived from gp100, MART-1, and tyrosinase emulsified in incomplete Freund's adjuvant, and present data to support the hypothesis that the progression of disease in this patient was due to in vivo immunoselection for immunoresistant tumor variants. The authors previously demonstrated the existence of T-cell clones in this patient's peripheral blood and tumor-infiltrating lymphocytes (TILs) reactive against multiple antigens, including gp100, the tyrosinase-related protein (TRP)-2, a novel TRP-2 isoform-TRP-2-6b, SOX10, and the melanoma antigen NY-ESO-1. In addition to the multiple HLA-A2 restricted T-cell clones, the authors have now identified additional HLA-B/C-restricted as well as class II (HLA-DP)-restricted anti-melanoma antigen T-cell clones from this patient's TIL. One recurrent tumor showed loss of expression of multiple tumor antigens but retention of HLA class I expression. The other recurrent lesion showed total loss of HLA class I expression even though the tumor cells still expressed many melanoma antigens. This paper thus provides evidence for both the effectiveness of the immune destruction of cancer as well as problems associated with antigen-loss tumor escape mechanisms.

Key Words: tumor-infiltrating lymphocytes, vaccination, melanoma antigens, tumor escape, immunoselection

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The identification of tumor-associated antigens (TAA) has opened new opportunities for the development of cancer immunotherapies. Most of the human melanoma antigens reported so far belong to two major groups: the melanocyte/melanoma-differentiation antigens (MDAs) and the cancer-testis antigens (CTA), such as MAGE and NY-ESO-1, that are overexpressed in tumors of various histologies but not in normal tissues except for testis. The commonly known MDAs include gp100, MART-1, tyrosinase, and the tyrosinase-related proteins TRP-1 and TRP-2. Many clinical vaccine trials have been conducted based on these antigens, using immunization with peptide epitopes with or without modification or with recombinant DNA vaccines. A recent study has suggested the efficacy of immunotherapy using a modified gp100 antigen together with high-dose interleukin-2 (IL-2).[1] Both humoral and cellular immune responses directed against NY-ESO-1 have been reported in patients with NY-ESO-1-expressing tumors.[2]

Despite recent progress in tumor immunobiology and technical advances in the field of tumor immunotherapy, current cancer vaccine strategies employed in the treatment of patients with cancer have been successful in only rare and sporadic cases. The lack of observed tumor regression is most likely due to inadequacies of current immunotherapy strategies. In some instances where objective clinical responses (i.e., complete and partial responses) have been observed after immunotherapy, tumors often recur or progress. It is not clear to what extent immunoselection for tumor variants that fail to express the appropriate antigens are responsible for these recurrences.

In this paper, we describe a patient who experienced progression and recurrence of melanoma tumors after an initial dramatic response to immunotherapy using peptides derived from gp100, MART-1, and tyrosinase emulsified in incomplete Freund's adjuvant (IFA), and present data to support the hypothesis that the progression of disease in this patient was due to in vivo immunoselection for immunoresistant tumor variants. We demonstrated the existence of T-cell clones reactive against multiple antigens in this patient's peripheral blood and tumor-infiltrating lymphocytes (TILs). This patient developed cellular immunity against 12 different MHC-restricted cancer antigens, including the HLA-A*0201 restricted gp100, TRP-2, a novel TRP-2 isoform-TRP-2-6b, the CTA NY-ESO-1, and the newly identified MDA SOX10,[3] as well as to HLA-B/C-restricted and class II (HLA-DP)-restricted melanoma antigens. Simultaneous loss of expression of multiple antigens or loss of expression of MHC molecules was seen that might have

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accounted for this patient's recurrence after a substantial clinical regression following immunotherapy.

MATERIALS AND METHODS

The patient was a 63-year-old woman who underwent a wide local excision of a primary melanoma on her back in 1981. In May 1997, she developed a subcutaneous metastasis on her left chest wall; it was resected, and she was started on a chemoimmunotherapy regimen comprising cisplatin, vinblastine, dacarbazine, IL-2, and interferon [IFN]-α. Because of disease progression, she was referred to the Surgery Branch, National Cancer Institute (Bethesda, MD) in June 1998. At that time, she had developed metastatic disease in multiple sites including the lungs, liver, intrapelvic area, left abdominal wall, left thigh, and subcutaneous (SQ) areas. She was started on a four-peptide vaccination protocol using 1 mg each of gp100: 209–217 (210M), gp100: 280–288 (288V), MART-1: 27–35, and tyrosinase: 368–376, emulsified in IFA SQ every 3 weeks. Most of her tumors completely regressed after two cycles of treatment (day 45), including complete resolution of a large tumor in her left thigh, an intrapelvic mass, a liver lesion, most of the nodules in her lungs, and all but one SQ lesion. She completed a total of six cycles of vaccinations in October 1998. The remaining SQ lesion was resected at the end of treatment (October 1998). A year later (October 1999), she developed a frontal lobe metastatic brain lesion; it was resected, together with a slow-growing subcutaneous nodule on her right chest wall. TIL 1790 used in this study was grown from the chest wall lesion. In March 2000, she underwent a second right temporal craniotomy for resection of recurrent disease at the prior brain resection site, followed by whole-brain irradiation. She continued to develop metastatic lesions at multiple sites, including the brain, and eventually died of disease in early 2002.

Cell Lines

Melanoma-reactive CTLs were derived from bulk TIL cultures grown in Iscove's modified Dulbecco medium (Gibco BRL, Gaithersburg, MD) containing 6,000 IU/mL of hrIL-2 (Chiron, Emeryville, CA). CTL clones were derived from bulk TIL cultures by limiting dilution with the addition of anti-CD3 antibody (Ab) (OKT-3, Ortho Pharmaceuticals, Raritan, NJ) as previously described.[4] Briefly, 5×10^4 irradiated (3,000 cG) peripheral blood mononuclear cells (PBMCs) from three allogeneic donors were plated in round-bottom 96-well plates with 0.5 to 90 T cells per well. Cells were cultured in RPMI 1640 medium (Gibco BRL) containing 20% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) and 30 ng/mL OKT-3 Ab with 300 IU/ml IL-2. The same dose of IL-2 was added on day 7, and clones were tested for recognition of HLA-A2$^+$ versus HLA-A2$^-$ melanoma cell lines 14 days after stimulation. After testing, the remainder of T cells were expanded by plating them in a T25 flask with 2.5×10^7 irradiated PBMCs from three allogeneic donors in 25 mL RPMI 1640 medium containing 10% FBS and 30 ng/mL OKT-3 Ab. Subsequent expansion was similarly done, but with 1 to 2×10^6 CTLs and 2.5×10^8 allogeneic PBMCs plated in an upright T162 flask in 150 mL medium.

All melanoma cell lines were established in the Surgery Branch (NCI) and previously described.[4] The autologous melanoma cell lines 1928 and 1973 were established from two recurrent SQ lesions resected in May and October 2001, respectively. T2 cells, deficient in transporter associated protein, were used to test HLA-A2-restricted peptides for CTL activity. All cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 10 mmol/L HEPES buffer, 100 U/mL penicillin-streptomycin (Biofluids), and 2 mmol/L L-glutamine (Biofluids).

Cytokine Release Assays

Five $\times 10^4$ CTL cells were plated with 1×10^5 target cells (tumor cells or T2 cells that had been pulsed with peptides at 1 μmol/L for 2 hours at 37°C and washed once) in 96-well round-bottom plates in 200 μL CM. After 18 to 24 hours of incubation at 37°C, the supernatant was harvested for detection of IFN-γ release using enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Cambridge, MA).

For the MHC blocking assays, target cells were incubated with the appropriate monoclonal antibody (mAb) at a final concentration of 50 μg/mL for 1 hour at 37°C prior to the addition of CTLs. W6/32 (anti-HLA class I), IVA12 (anti-HLA class II), and HB55 (anti-HLA-DR) were obtained from ATCC. Anti-HLA-DP and DQ (clones B7/21 and 1a3, respectively) were obtained from Leinco (St. Louis, MO).

RT-PCR Assays for Tumor Antigen Expression

Total RNA of melanoma samples was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was prepared from total RNA using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Primers used for PCR were as follows: gp100 (forward) 5′-GCTTGGTGTCTCAAGGCAACT-3′ and (reverse) 5′-CTCCAGGTAAGTATGAGTGAC-3′, PCR product size: 751 bp[5]; MART-1 (forward) 5′-ATGCCAAGAGAAGATGCTCAC-3′ and (reverse) 5′-AGCATGTCTCAGGTGTCTCG-3′, PCR product size: 384 bp[5]; tyrosinase (forward) 5′-TTGGCAGATTGTCTGTAGCC-3′ and (reverse) 5′-GCTATCCCAGTAAGTGGACT-3′, PCR product size: 252 bp[5]; NY-ESO-1 (forward) 5′ AGCCGCCTGCTTGAGTTCTACCTC 3′ and (reverse) 5′ AGGGAAAGCTGCTGGAGACAG 3′ PCR product size: 221bp[6]; SOX10 (forward) 5′-CTTCGGCAACGTGGACATT-3′ and (reverse) 5′-TCAGCCACATCAAAGGTCTCC-3′, PCR product size: 72 bp; TRP-2 and TRP-2-6b (forward) 5′- ACTGCGAGCGGAAGAAACCA-3′ and (reverse) 5′-GGCATCTGCAGGAGGATTAA-3′ and 5′-ATGCAGGGAAGGGAGTTCCT-

TABLE 1. HLA-A2-Restricted Activities of Patient's TIL Clones and Cloids

		T-Cell Clones or Cloids from Patient								
		M26	M-A1	M18	M-E5	M-D2	M-G5	M8	M37	MR1
Targets	HLA-A2	(pg/mL IFN-γ)								
624mel	+	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	59
526mel	+	>1000	>1000	>1000	>1000	>1000	>1000	0	>1000	2
F002Rmel	+	1	0	24	0	>1000	>1000	>1000	>1000	>1000
1102mel	+	>1000	>1000	303	>1000	>1000	226	0	>1000	11
624-28mel	−	0	0	1	0	2	0	0	2	7
397mel	−	0	0	1	2	2	0	0	0	4
938mel	−	0	0	0	2	2	0	0	0	0
T2 pulsed with										
gp100:154-162	+	0	0	0	>1000	3	0	0	0	0
gp100:209-217	+	>1000	0	0	1	2	0	0	0	0
gp100:280-288	+	0	0	0	0	1	0	0	0	3
MART-1:27:35	+	0	>1000	>1000	>1000	0	0	0	0	7
TYR:368-376	+	0	0	761	0	0	0	0	0	11
TRP-2:180-188	+	0	0	4	5	>1000	1	0	0	4
TRP-2-6b:403-411	+	0	2	7	9	5	>1000	1	0	4
NY-ESO-1:157-165	+	0	1	6	6	7	2	>1000	0	5
SOX10:331-340	+	0	1	7	8	7	4	0	>1000	0
T cell alone		0	1	6	7	7	4	0	0	4
Antigen recognized		gp100 (209-217)	MART-1	MART-1; Tyrosinase	gp100 (154-162); MART-1	TRP-2	TRP-2-6b	NY-ESO-1	SOX10	Unknown

3', respectively, PCR product size: 998 bp and 923 bp, respectively; β-actin (forward) 5'-ACACTGTGCCCATCTACGAGG-3' and (reverse) 5'-AGGGGCCGGACTCGTCATACT-3', PCR product size: 621 bp[7]; β2-m (forward) 5'-ATTCGGGCCGAGATGTC-3' and (reverse) 5'-ACCTCCATGATGCTGCTTACA-3', PCR product size: 388 bp. Cycling conditions were 94°C for 1 minute, 60°C for 30 seconds, and 72°C for 2 minutes for 35 cycles, except for β-actin (31 cycles).

TABLE 2. Activities of Non-HLA-A2-Restricted TIL Clones

	Clones	
	M-D5	M-E10
Targets	(pg/mL IFN-γ)	
624-28mel	3	42
624mel	2	132
888mel	7	58
888A2mel	8	52
938mel	10	46
526mel	1	>1000
SK23mel	5	65
1973mel	9	53
1928mel	>1000	52
1938mel	9	457
Medium	1	52

TABLE 3. HLA-Blocking Assay of Clones M-E10 and M-D5

			Controls†		
	M-E10	M-D5	M-A1	LB7	LB11
Target* Plus	(pg/mL IFN-γ)				
No mAb	940	1009	860	484	606
Anti-HLA class I	0	1	0	336	13
Anti-HLA A2	839	765	30	446	536
Anti-HLA B/C	0	34	610	419	8
Anti-HLA class II	788	657	659	64	440
T cell alone	0	0	0	3	4
% reduction	100	97	97	87	99

*Targets: 526 mel for M-E10 and M-A1; 1928 for M-D5; F002Rmel for LB7 and LB11.

†Controls: M-A1 is an HLA-A2-restricted, MART-1-specific CTL clone. LB7 and LB11 are two allogeneic HLA-DR- and HLA-B/C-restricted clones, respectively.

cDNA Sequencing to Detect Mutations in β2-m

Sequencing of β2-m cDNA derived from 1973 melanoma cells was performed with an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA) using the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). Mutations were searched against the GenBank database using the BLAST algorithm and Global Alignment of Two Sequences.

RESULTS

Activities of TIL Clones/Cloids Derived From Bulk TIL 1790

TIL line 1790 was isolated from a slow-growing SQ lesion resected from the patient's right chest wall in November 1999. In an attempt to identify the antigen(s) recognized by TIL 1790, a number of TIL clones/cloids were generated from this TIL line by limiting dilution. The activities of the HLA-A2-restricted TIL clones and cloids against melanoma cell lines and multiple peptide antigens are shown in Table 1. Two clones with separate HLA-B- or C-restricted activities were also isolated from the bulk TIL (Tables 2 and 3). In addition, the patient's pretreatment PBMCs also showed strong preexisting immunity to gp100 (epitope 154–162), the two TRP-2 antigens, and NY-ESO-1 (data not shown).

Besides the class I-restricted reactivities, the bulk TILs also contained a $CD4^+$ population that recognized tumors in a class II HLA-DP-restricted fashion (data not shown).

Loss of Recognition by Autologous TIL Clones of Autologous Melanoma Cell Lines Established From Recurrent Lesions

Recurrent subcutaneous tumors on the right upper abdomen and right lower back lesions were resected in May and October 2001, respectively. Melanoma lines, 1928mel and 1973mel, respectively, were established in vitro from the two lesions and were used as targets for recognition by autologous TIL clones. Neither cell line was recognized by autologous HLA-A2-restricted, antigen reactive TIL clones that recognized gp100, MART-1, TRP-2, TRP-2-6b, or NY-ESO-1 epitopes (Table 4).

Characterization of Autologous Melanoma Lines 1928mel and 1973mel

To elucidate the mechanisms responsible for the lack of recognition of melanoma lines 1928mel and 1973mel by autologous HLA-A2-restricted TIL clones, RT-PCR analysis was performed to check for expression of known melanoma antigens. In addition, expression of HLA class I and HLA-A2 antigens on the two cell lines was analyzed by FACS. 1928mel expressed both HLA class I and HLA-A2 (Fig. 1) but did not express any known melanoma antigens tested except for weak expression of the MDA SOX10 (Fig. 2).

In contrast, 1973mel expressed all known melanoma antigens tested except for NY-ESO-1 (see Fig. 2) but did not express HLA class I antigens (see Fig. 1). A functional peptide-pulsing assay was performed to confirm the findings. 1928mel, but not 1973mel, when pulsed with an HLA-A2 peptide (MART-1: 27–35) was recognized by a MART-1-specific TIL clone (Table 5), further demonstrating the lack of HLA-A2 expression by 1973mel.

TABLE 4. No Recognition of Autologous Cell Lines, 1928mel and 1973mel, by Autologous HLA-A2-Restricted TIL Clones

	TIL Clones from Patient				
	MB4	M6	MR7	M8	M26
Targets	(pg/mL IFN-γ)				
938mel*	7	8	11	40	36
624mel†	>1000	>1000	>1000	>1000	>1000
1928mel	32	41	41	11	11
1973mel	41	43	43	16	7
Medium	42	23	14	15	4
Antigen recognition	TRP-2-6b	MART-1	TRP-2	NY-ESO-1	gp100

*HLA-A2 positive control melanoma cell line.
†HLA-A2 negative control melanoma cell line.

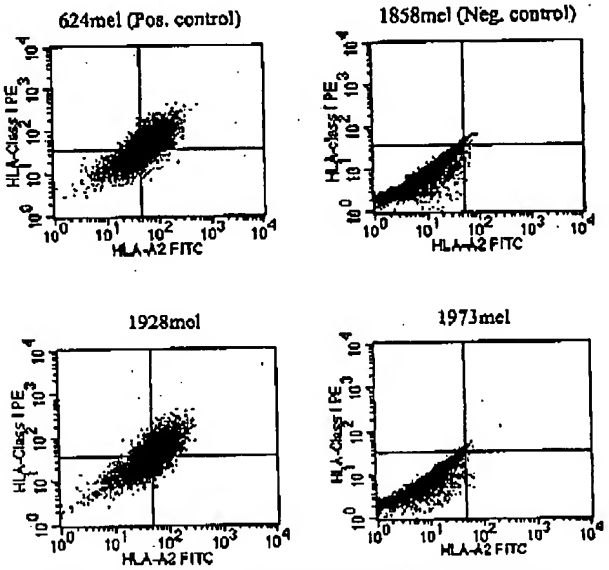
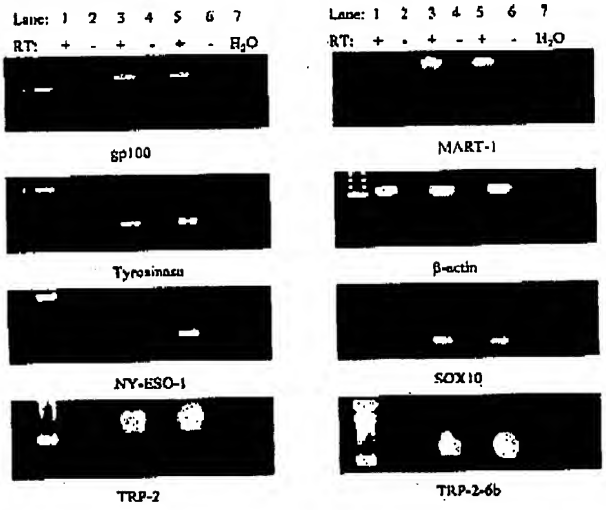


FIGURE 1. HLA class I and HLA-A2 expression of the two autologous cell lines, 1928mel and 1973mel.



Melanoma cell line	Melanoma antigens						
	gp100	MART-1	Tyrosinase	NY-ESO-1	SOX10	TRP-2	TRP-2-6b
1928mel	-	-	-	-	+	-	+
1973mel	+	+	+	-	+	+	+
624mel	-	+	+	+	+	+	+

FIGURE 2. Melanoma antigen expression patterns in the two autologous cell lines, 1928mel and 1973mel, derived from two recurrent lesions. The allogeneic cell line 624mel, which expressed all antigens of interest, was used as a positive control. Lanes 1 and 2: 1928mel; lanes 3 and 4: 1973mel; lanes 5 and 6: 624mel; lane 7: no template control (H_2O control)

Mutation of β_2m in 1973mel Resulted in Loss of MHC Class I Expression

Since IFN-γ-treated 1973mel was not recognized by autologous HLA-A2-restricted TIL clones (data not shown), the mechanism underlying total loss of MHC class I antigens in 1973mel was more likely a result of structural rather than functional alterations. From our previous studies[8] and recent unpublished observations, loss of β_2m (due to somatic mutations) was demonstrated in some melanoma cell lines that exhibited total loss of MHC class I expression. Therefore, the β_2m cDNA derived from 1973mel was sequenced and found to have a 2 base-pair (TT) deletion at positions 331–332, resulting in a truncated protein secondary to an early stop codon (Fig. 3). To confirm that the mutated β_2m was the underlying cause of total loss of MHC class I antigens, 1973mel was electroporated with a plasmid encoding the wild-type (WT) β_2m cDNA and tested for recognition by autologous HLA-A2-restricted TIL clones. Only 1973mel transfected with WT β_2m but not the control GFP plasmid was recognized (Table 6).

TABLE 5. Functional Assay to Test for HLA-A2 Expression by Autologous Cell Lines 1928mel and 1973mel

Targets	Pulsed with	M6* (pg/mL IFN-γ)	LB7.4.2† (pg/mL IFN-γ)
1928mel	None	10	969
1928mel	gp100:280–288	14	931
1928mel	MART-1:27–35	687	953
1973mel	None	5	728
1973mel	gp100:280–288	10	723
1973mel	MART-1:27–35	6	507
T2	None	6	12
T2	gp100:280–288	7	11
T2	MART-1:27–35	923	11
Medium		9	8

*M6 is an HLA-A2-restricted, MART-1-specific CTL clone.
†LB7.4.2 is a control allogeneic CD4+ clone that recognized 1928mel and 1973mel.

Earlier Tumor Recognized by Autologous HLA-A2-Restricted TIL Clones

To test whether the loss of recognition of the two recurrent tumors, 1928mel and 1973mel, was a later event during the patient's disease progression, an earlier tumor (FrTu 1790), resected approximately 1.5 years before lesions 1928 and 1973, was used to test for recognition by autologous HLA-A2-restricted TIL clones. Since we could not establish a cell line from this lesion, a fresh tumor cell suspension was used as a target. FrTu 1790 was recognized by multiple TIL clones (Table 7). This functional assay demonstrated that many of the cells in FrTu 1790 expressed both HLA-A2 antigen and multiple melanoma-associated antigens.

DISCUSSION

Current cancer vaccine strategies have been only sporadically successful in eradicating tumors, probably mainly due to the inadequacies of current immunization regimens. A few patients have experienced dramatic clinical responses following immunization with cancer antigens, although tumors recur in many of these patients. Partial or total loss of HLA class I expression or downregulation of tumor antigens in the residual or recurrent tumors has been demonstrated in some cases, thus accounting for the escape of tumor from immune destruction.[9–11]

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1973mel's β2m:        GAACCATGTGAC..TGTCACAG
                      ||||||||||| ||||||||
Wild-type β2m:  319 GAACCATGTGACTTGTCACAG 340
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FIGURE 3. Double base-pair deletion (underlined) detected in the β_2m cDNA derived from melanoma cell line 1973mel. Wild-type β_2m was from GenBank sequence NM_004048.1.

TABLE 6. Recognition of 1973 Melanoma Cells Transfected with Wild-Type β2m by Autologous HLA-A2-Restricted TIL Clones

Targets	HLA-A2	M8	M-A1	M28	M37
		(pg/mL IFN-γ)			
1973mel/GFP		0	15	1	0
1973mel/β2m		0	438	>1000	137
624-28mel	−	0	0	0	0
624mel	+	943	>1000	>1000	>1000
Medium		3	7	1	4
Antigen recognized		NY-ESO-1	MART-1	TRP-2-6b	SOX10
Antigen expressed in 1973mel		−	+	+	+

This article describes immune studies of a unique patient who exhibited a dramatic regression of cancer at multiple visceral and soft tissue sites following immunization with four HLA-A2 peptides from three MDAs—gp100, MART-1, and tyrosinase—emulsified in IFA. Most of her tumors completely regressed after two cycles of treatment (day 45), including complete resolution of a large tumor in her left thigh, an intrapelvic mass, a liver lesion, most of the nodules in her lungs, and all but one SQ lesion. We demonstrate here that tumor recurrence was associated with loss of either melanoma antigens or MHC molecules.

Evidence is presented here for in vivo immunoselection of NY-ESO-1-negative tumor variants. Tumor 1790 (year resected, 1999) was recognized by an NY-ESO-1-specific TIL clone (M8). However, both recurrent tumors in 2001–tumors 1928 and 1973–were negative for NY-ESO-1 expression by RT-PCR, and neither was recognized by clone M8. The patient had strong preexisting immunity to NY-ESO-1 as detected in her pretreatment PBMCs,[4] even though she was not vaccinated against NY-ESO-1 antigen. In addition, NY-ESO-1-specific lymphocytes have also been cloned from her TILs.

In addition, the patient's TILs and peripheral blood contained reactivities directed against multiple melanoma antigens, including gp100: 209–217, gp100: 154–162, MART-1, tyrosinase, TRP-2, TRP-2-6b, NY-ESO-1, SOX10, an as-yet-known HLA-A2-restricted antigen, two HLA-B- or C-restricted antigens, as well as an HLA-DP-restricted antigen. These multiple immune reactivities may account for the emergence of immunoresistant tumor variant 1928, which lost expression of most of the known antigens mentioned previously. Even though cell line 1928mel expressed SOX10, the level of expression was minimal, as demonstrated by a weaker band on a highly sensitive RT-PCR assay (see Fig. 2), and by real-time quantitative RT-PCR.[3] Even though downregulation of individual antigens due to separate mutational events is a possibility, regulatory mechanisms such as cytokine-driven antigen silencing[12] seem to be a more plausible explanation. Since SOX10 is a potent transactivator of the microphthalmia-associated transcription factor (*MITF*) gene,[13] which in turn regulates the expression of multiple MDAs,[14,15] downregulation of SOX10 through regulatory mechanisms may interfere with the expression of multiple MDAs. Therefore, we hypoth-

TABLE 7. Recognition of an Earlier Tumor (FrTu 1790) by Autologous HLA-A2-Restricted TIL Clones

Targets	HLA-A2	TIL Clones from Patient					
		M26	M-A1	M-D2	M-G5	M8	M37
		(pg/mL IFN-γ)					
FrTu 1790		261	751	847	803	>1000	318
1928mel	+	0	45	10	24	5	369
1973mel	−	0	0	14	21	6	0
624mel	+	>1000	>1000	>1000	494	770	>1000
624-28mel	−	0	2	12	5	4	0
Medium		0	0	35	0	0	0
Antigen recognized		gp100	MART-1	TRP-2	TRP-2-6b	NY-ESO-1	SOX10

esize that the loss of antigen expression was a result of two separate events: one involved the loss of the CTA NY-ESO-1, most likely by a mutation, and the other involved SOX10 and other MDAs, most likely through a regulatory mechanism.

Another immunoresistant phenotype that emerged from this patient's recurrent tumors was a total HLA class I loss variant. Tumor 1973, which recurred in October 2001, expressed multiple melanoma antigens but did not express HLA class I. The mechanism underlying this phenotype was a double base-pair deletion in the β-2m molecule. This accounts for the lack of recognition of 1973mel by HLA-A2-restricted autologous TIL clones and helps explain the recurrence and progression of tumor 1973.

The fact that autologous HLA-A2-restricted TIL clones specific for multiple melanoma antigens recognized an earlier tumor (FrTu 1790) resected close to 2 years before the resection of the two recurrent lesions is an indication that FrTu 1790 expressed both HLA-A2 antigen and multiple melanoma antigens. Therefore, it seems that in vivo immunoselection of immunoresistant tumor variants that exhibited total loss of HLA-class I antigens or multiple melanoma antigens indeed occurred in this patient, who experienced a dramatic response but later died of recurrent disease. The appearance of FrTu 1790 recurrent lesion in the presence of multiple tumor-specific T-cell clones 1 year after treatment may reflect the suboptimal activation state of these T cells without help from exogenous sources such as the peptide/IFA vaccination used in the previous treatment. Recurrent tumors 1928 and 1973 most likely originated from a few immunoresistant tumor cell clones that escaped immune destruction during the peptide/IFA treatment. It is likely that these tumor cell clones might have taken a few years to become clinically detectable, as seen in 2001 when they were resected.

In conclusion, this paper illustrates a situation that may become more prevalent as immunotherapy-based treatments for cancer become more effective: the destruction of immunosensitive tumor cells but the eventual progression of immunoresistant tumor variants.

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HER-2/neu antigen loss and relapse of mammary carcinoma are actively induced by T cell-mediated anti-tumor immune responses

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Induction of tumor-specific immune responses results in the inhibition of tumor development. However, tumors recur because of the tumor immunoediting process that facilitates development of escape mechanisms in tumors. It is not known whether tumor escape is an active process whereby anti-tumor immune responses induce loss or downregulation of the target antigen in the antigen-positive clones. To address this question, we used rat neu-overexpressing mouse mammary carcinoma (MMC) and its relapsed neu antigen-negative variant (ANV). ANV emerged from MMC under pressure from neu-specific T cell responses *in vivo*. We then cloned residual neu antigen-negative cells from MMC and residual neu antigen-positive cells from ANV. We found marked differences between these neu-negative clones and ANV, demonstrating that the residual neu-negative clones are probably not the origin of ANV. Since initial rejection of MMC was associated with the presence of IFN-γ-secreting T cells, we treated MMC with IFN-γ and showed that IFN-γ could induce downregulation of neu expression in MMC. This appears to be due to methylation of the neu promoter. Together, these data suggest that neu antigen loss is an active process that occurs in primary tumors due to the neu-targeted anti-tumor immune responses.

Key words: Cytokines · Immune evasion · Tumor immunology

Introduction

Tumor escape and recurrences are major challenges in immunotherapy of cancers, including breast carcinomas. Therefore, understanding the mechanisms by which primary tumors escape from the host immune responses may offer critical insights into the improvement of cancer immunotherapy and lead to the development of new immunotherapeutic approaches. A variety of molecular alterations in tumors have been reported. These include, but are not limited to, the loss or downregulation of MHC class I antigens in tumors, defects in antigen presentation machinery such as TAP and/or β-2 microglobulin, expression of Fas ligand and/or loss of Fas in tumors, expression of HLA-E or Qa1 as killer inhibitory ligands in tumors, and loss of tumor antigens [1–4].

While immune responses can be induced against a variety of cancers, resulting in the inhibition of tumor development, molecular alterations in tumors can also occur under immune pressure, resulting in tumor escape. In other words, anti-tumor immune responses

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Abbreviations: ANV: neu antigen-negative variant · **MMC:** neu overexpressing mouse mammary carcinoma · **MMTV:** mouse mammary tumor virus

can function as a "double-edged" sword exerting both host-protective and tumor-evading effects on developing cancers; "cancer immunoediting" has been coined to accurately describe the latter phenomena. It was reported that chemically induced methylcholanthrene (Meth A) sarcomas derived from immunocompetent animals were more tumorogenic when inoculated into naive wild-type mice than tumors similarly derived from immunodeficient animals [5–7]. Based on these findings, it has been envisaged that cancer immunoediting is a result of three processes: elimination, equilibrium, and escape [8]. At the equilibrium phase between immune response and tumor growth, Darwinian selection has been suggested to be the mechanism for cancer immunoediting [9]. According to Darwinian selection, the tumor-specific immune responses eliminate highly immunogenic tumor cells, leaving behind tumor variants of reduced immunogenicity that have a better chance of surviving in the immunocompetent host. There are also reports suggesting that immune-mediated induction of epigenetic changes in primary tumors leads to tumor antigen loss [10, 11]. Using a neu-overexpressing primary tumor (mouse mammary carcinoma, MMC) and its relapsed neu antigen-negative variant (ANV), we describe how neu-specific immune responses may induce tumor escape. We show that IFN-γ is involved in downregulation of neu expression in primary tumors by inducing methylation of the mouse mammary tumor virus (MMTV) promoter.

Results

Rejection of MMC in FVB mice is mediated by host T cell responses

Wild-type FVB mice spontaneously reject MMC because the tumor cells overexpress rat neu antigen. In order to determine whether spontaneous rejection of MMC in FVB mice is mediated by T cells, animals were depleted of $CD4^+$ and $CD8^+$ T cells by the injection of GK1.5 and 2.43 Ab, respectively [12]. Animals were then inoculated with MMC (5×10^6 cells/mouse). Depletion of T cell subsets continued until the end of the trial. As shown in Fig. 1A, all wild-type mice rejected MMC within 3 wk, while animals depleted of $CD4^+$ and $CD8^+$ T cells ($CD4^-/CD8^-$) progressively developed tumors. In order to determine the neu specificity of the tumor

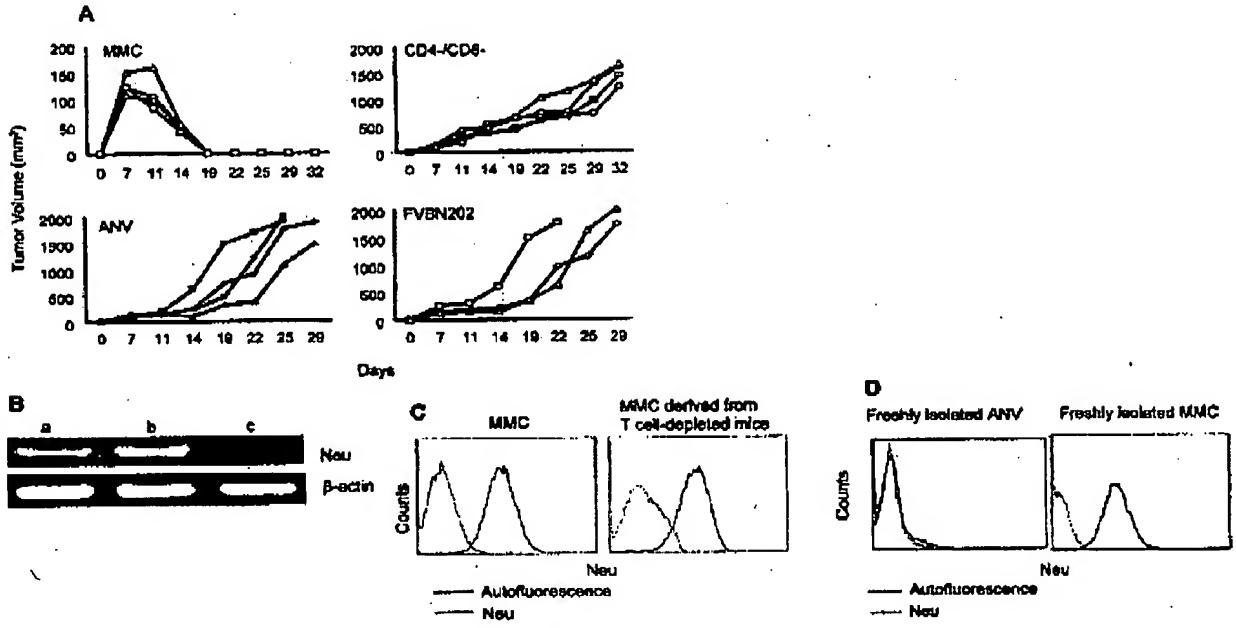

Figure 1. Rejection of MMC in FVB mice and downregulation of neu antigen are mediated by T cell immune responses. (A) Wild-type immunocompetent (MMC) or $CD4^+$ and $CD8^+$ T cell-depleted ($CD4^-/CD8^-$) FVB mice ($n=4$) were inoculated with MMC. One group of FVB mice was inoculated with ANV (ANV). As a control, FVBN202-transgenic mice ($n=3$), which are tolerant to neu protein and fail to reject MMC, were inoculated with MMC (FVBN202). (B) RT-PCR analysis for detection of neu mRNA in tumors derived from MMC-challenged $CD4^-/CD8^-$ FVB mice (a) show no neu antigen loss. Expression of neu mRNA in neu-overexpressing MMC (b) and neu-negative ANV (c) was determined as positive and negative controls. (C) Detection of neu expression by flow cytometry analysis of a viable MMC line and MMC cells isolated from $CD4^-/CD8^-$ FVB mice. (D) Expression of the neu protein in freshly isolated ANV or MMC.

rejection, wild-type mice were inoculated with ANV. All the mice failed to reject ANV. As an additional control for the neu specificity of MMC rejection, FVBN202-*transgenic* mice that tolerate neu protein were inoculated with MMC. All the FVBN202 mice progressively developed tumors. Semi-quantitative RT-PCR analysis of the tumors isolated from $CD4^-/CD8^-$ FVB mice showed no neu antigen loss in the absence of effector T cells (Fig. 1B). The MMC cell line and the relapsed ANV tumors were used as neu-positive (b) and neu-negative (c) controls, respectively. Expression of neu mRNA in the MMC cell line was higher (100%) than expression in freshly isolated mRNA from solid tumors (61%) because of the presence of other infiltrating cells in the tumor microenvironment. When these solid tumors were cultured *in vitro* to establish viable tumor clones, neu overexpression in the clones was consistently comparable with neu overexpression in the MMC line (MFI: 276 *versus* 265, respectively; Fig. 1C). Freshly isolated MMC or ANV were positive or negative for neu expression, respectively (Fig. 1D).

Heterogeneity of MMC and ANV in the expression of neu

We have established several lines of primary MMC and relapsed ANV cells. Flow cytometry analyses of MMC and ANV lines showed residual neu-negative clones among MMC cells and neu-positive clones among ANV (Fig. 2A). We sought to determine whether ANV emerged from the residual neu-negative clones in MMC because of the elimination of neu-overexpressing clones by anti-neu immune responses. We sorted neu-negative clones of MMC (MMC^{neg}) and neu-positive clones of ANV (ANV^{pos}); MMC^{neg} cells were 100% neu-negative, while ANV^{pos} cells were 100% neu-positive, with intermediate expression of neu as compared to MMC (Fig. 2A). These cells were stable for the absence or presence of neu expression over 45 passages. Semi-quantitative RT-PCR analysis also showed that ANV^{pos} and MMC^{neg} cells were positive (23%) and negative (0%), respectively, for the expression of neu mRNA (Fig. 2B). MMC had the highest expression of neu mRNA (100%), while ANV had minimal neu expression (5%). Since neu expression in the FVBN202-*transgenic* mouse is regulated by hypomethylation of MMTV promoter [13], we used bisulfite methylation assays and detected hypomethylation and hypermethylation of the CpG-rich sites within region 1 (corresponding to nucleotides 56–316) of MMTV in MMC or ANV^{pos} and ANV or MMC^{neg}, respectively (Fig. 2C). There was no mutation in the neu gene or MMTV promoter (data not shown).

Distinct proliferation rates of ANV and MMC^{neg} clones

In order to determine proliferation rates *in vitro*, all the tumor lines (2.5×10^5 cells) were cultured for 3 days, and viable cells were counted in triplicates using trypan blue exclusion. The viability of cells was above 98%. Proliferation rates were calculated as follows: total cell numbers after 3-day culture divided by cell numbers on day 0. As shown in Fig. 3A, ANV had the highest rates of proliferation (8.8), while ANV^{pos} had the lowest rates (2.6). Proliferation rates of MMC (5) and MMC^{neg} (4.5) were comparable. Student's *t*-test analysis showed significant differences in proliferation rates between ANV and MMC^{neg} ($p=0.01$) or between MMC and ANV^{pos} ($p=0.03$).

Differential expression of $H\text{-}2^q$, STAT-1 and Fas in ANV and MMC^{neg} clones

There were substantial differences between MMC and ANV, ANV^{pos} and MMC, or MMC^{neg} and ANV in downregulation and upregulation of certain genes (shown in gray dots) (Fig. 3B). Since initial rejection of MMC in FVB mice was mediated by T cells, we evaluated expression of functional genes such as $H\text{-}2^q$, the IFN-γ downstream signaling molecule STAT-1, and Fas in these tumor lines using three biological replicates. As shown in Fig. 3C, ANV^{pos} had higher levels of $H\text{-}2^q$ expression (MFI: 67) compared to MMC (MFI: 15) ($p=0.007$). MMC^{neg} appeared to have lower levels of $H\text{-}2^q$ (MFI: 14) compared to ANV (MFI: 30) ($p=0.03$). Staining of the tumor cells was performed at the same time, and there was no variation in autofluorescence. Therefore, MFI are presented after the subtraction of the autofluorescence.

Semi-quantitative RT-PCR analyses showed that expression of STAT-1 was higher in ANV^{pos} (95.4%) and MMC^{neg} (100%) cells than in MMC (45.9%) and ANV (18.6%) cells. While MMC showed higher expression of STAT-1 than ANV, expression of Fas was higher in ANV (64.2%) than in MMC (38.5%) cells. ANV^{pos} (96.3%) and MMC^{neg} (100%) had higher levels of Fas than MMC or ANV (Fig. 3D).

IFN-γ-induced methylation of the MMTV promoter downregulates neu expression in MMC

Initial rejection of MMC in FVB mice occurred in the presence of MMC-specific IFN-γ-secreting T cells, while splenocytes of FVBN202-transgenic mice showed no MMC-specific IFN-γ secretion (Fig. 4A). MMC and ANV appeared to have similar levels of expression of IFN-γ receptor [14]. Therefore, we cultured MMC in the presence or absence of IFN-γ *in vitro* to determine

whether IFN-γ may induce neu antigen loss. At the end of day 3, the viability of adherent MMC was above 99%, as determined by trypan blue exclusion, and the total number of cells reached 21.7 × 10^6 in the absence of IFN-γ. IFN-γ induced apoptosis in the majority of MMC, so that total cell numbers reached 9.38 × 10^6, with only 28% viability (2.6 × 10^6 viable MMC). Flow cytometry analyses of the viable cells showed that IFN-γ increased early and late apoptotic cells from 14% and 15% to 35% and 28%, respectively (Fig. 4B). IFN-γ-treated viable MMC showed downregulation of neu expression both at the mRNA (Fig. 4C) and the protein (MFI: 329 *versus* 154; Fig. 4D) level after a 3-day culture. Such effects were abolished when the IFN-γ receptor was blocked using GR20 Ab (Fig. 4D). Bisulfite genomic sequencing showed that downregulation of neu expression by IFN-γ was due to IFN-γ-induced methylation of the MMTV promoter in MMC (Fig. 4E). In order to evaluate the status of neu expression *ex vivo* at the time of MMC rejection, tumors were removed 10–14 days after the challenge. Flow cytometry analyses of MMC showed downregulation of neu expression. However, 3- to 4-wk cultures of these MMC in the absence of IFN-γ resulted in upregulation of neu expression *ex vivo* (Fig. 4F). In

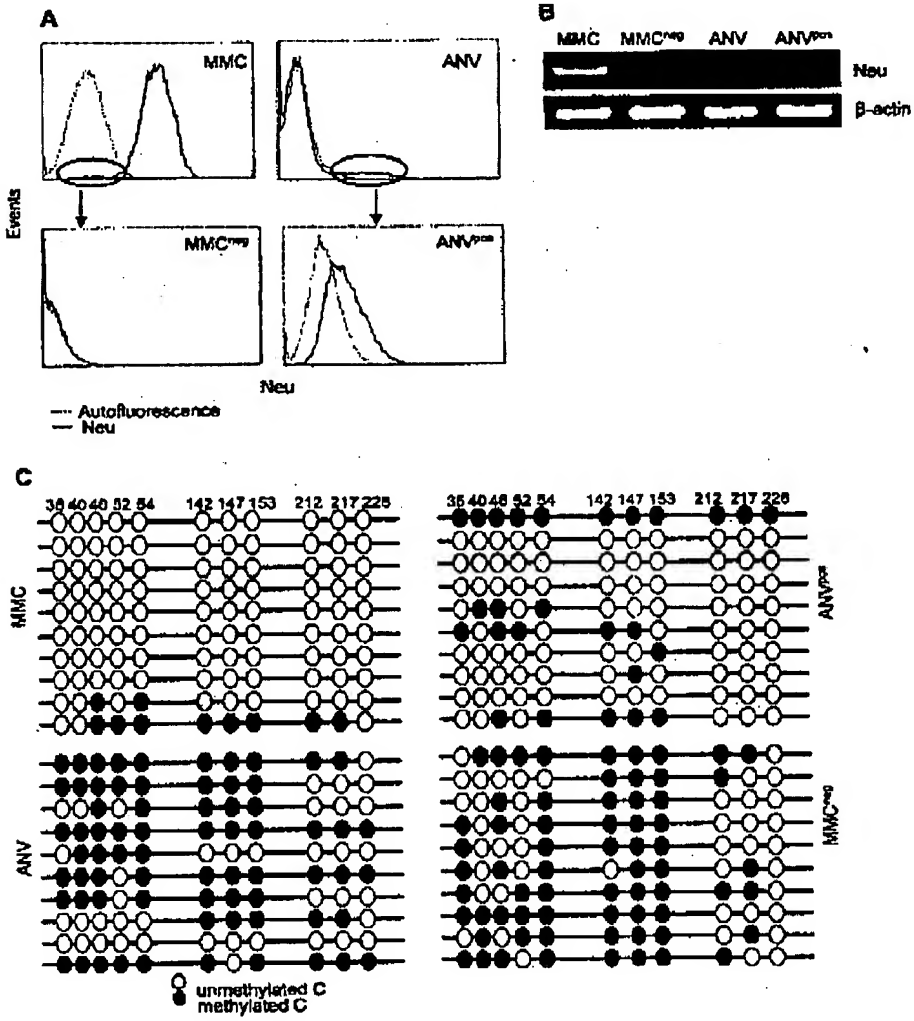

Figure 2. Establishment of MMC^{neg} and ANV^{pos} tumor lines from MMC and ANV. (A) MMC or ANV were stained with anti-neu Ab and subjected to flow cytometry analyses. Residual neu-negative clones of MMC and neu-positive clones of ANV were sorted using the Beckman Coulter EPICS Elite sorter. Sorted cells were cultured and cloned in vitro and subjected to flow cytometry for further analyses of neu expression. (B) RT-PCR analysis of the indicated tumor lines for the expression of neu mRNA using β-actin as an internal control. (C) Bisulfite genomic sequencing of the indicated cell lines. A total of ten clones per sample was sequenced.

order to determine whether hypomethylation of the MMTV promoter in ANV may reverse neu expression, we treated ANV with 1 and 3 μM 5-aza-deoxycytidine for 3 days. As shown in Fig. 4G, 3 μM 5-aza-deoxycytidine reversed neu expression in ANV as determined by RT-PCR. A higher concentration of 10 μM 5-aza-deoxycytidine was toxic and failed to reverse neu expression in ANV (data not shown).

In order to determine the role of IFN-γ in the downregulation of neu expression in MMC *in vivo*, we used two clones of MMC: IFN-γ receptor-positive MMC ($MMCr^+$) and IFN-γ receptor-negative MMC ($MMCr^-$). Heterogeneity of MMC in the expression of IFN-γ receptor alpha allowed us to prepare $MMCr^+$ and $MMCr^-$ clones through cell passages *in vitro*. Freshly isolated MMC from spontaneous mammary tumors were $MMCr^+$, but they lost expression of IFN-γ receptor after a number of passages *in vitro* and became $MMCr^-$. However, expression of neu antigen remained intact in $MMCr^-$. In order to eliminate the role of Ab responses in the rejection of MMC and focus on $CD8^+$ T cells as a major source of the neu-specific IFN-γ production, we depleted $CD4^+$ T cells *in vivo* at the priming phase of the immune response. Animals were then inoculated with $MMCr^+$ or $MMCr^-$ (Fig. 5A). While helpless $CD8^+$ T cells rejected $MMCr^-$ aggressively, $MMCr^+$ remained at a plateau until 2–3 months after the challenge and then grew aggressively (Fig. 5B). Aggressive growth of

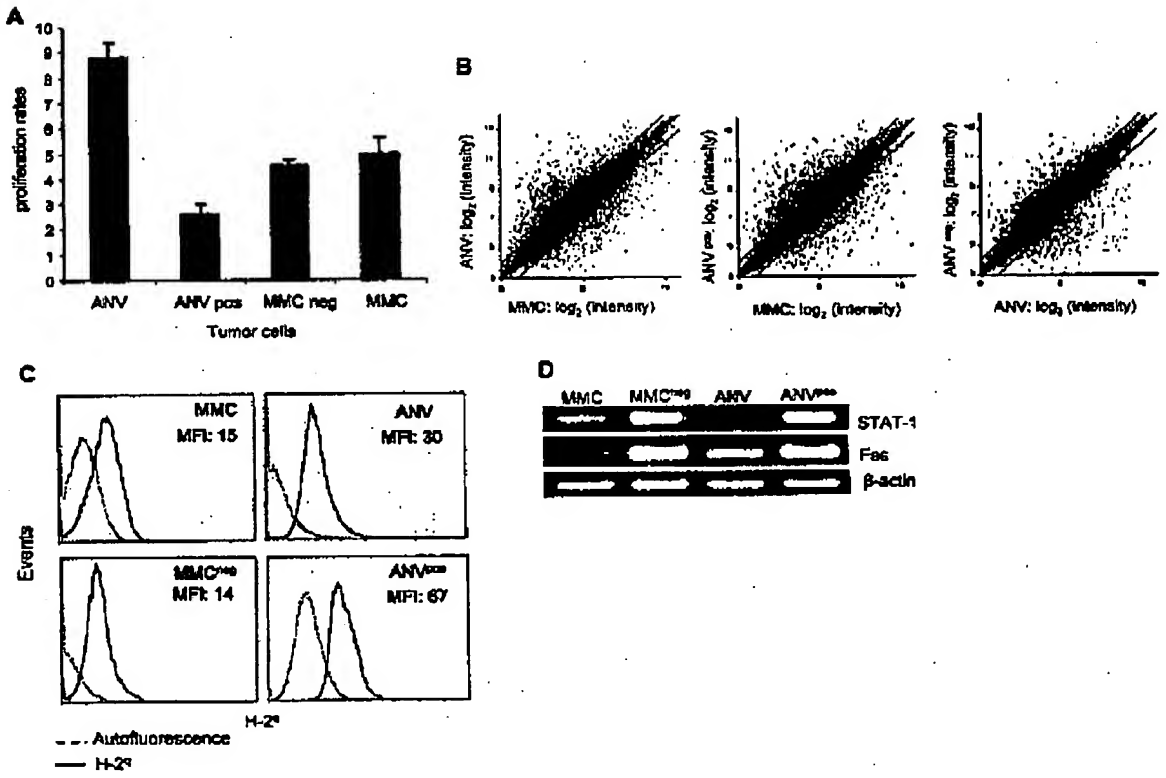

Figure 3. MMC and ANV appear to have distinct morphology, proliferation rates, and gene expression profiles. (A) All the tumor lines were cultured at 2.5×10^5 cells/well in triplicates using tissue culture dishes. After 3 days in culture, adherent cells were detached using 0.25% Trypsin-EDTA. Cells were then counted using trypan blue exclusion. Proliferation rates were calculated as follows: total cell numbers after 3 days in culture divided by cell numbers on day 0. (B) Microarray analysis was performed on the indicated samples. Background correction, normalization, and expression summaries were calculated. Scatter plot of log2-transformed expression summaries of the 22 690 probe sets in the Mouse430A 2.0 array are plotted for the samples indicated in the axes of the graphs. Gray dots show genes that were at least 2-fold different for MMC *versus* ANV, MMC *versus* ANV^{pos}, or ANV *versus* MMC^{neg}. Similar results were obtained in independent experiments using two different microarray analyses on biological replicates of cells. (C) The indicated tumor lines were subjected to flow cytometry-based analyses using mouse anti-H-2^d and FITC-conjugated anti-mouse Ig Ab. Isotype control Ab showed MFI similar to the autofluorescence (data not shown). Representative histograms are presented, and the MFI of quadruplicate experiments are shown after subtraction of the autofluorescence. (D) RT-PCR analysis of STAT-1 and Fas mRNA isolated from MMC, MMC^{neg}, ANV, and ANV^{pos}. Expression of β-actin was determined as an internal control.

MMCr$^+$ was associated with the loss of neu expression and progression of MMC into ANV, while neu expression remained unchanged during the plateau phase (Fig. 5B). MMCr$^-$ did not relapse during this follow-up period. Rejection of MMCr$^-$, but not MMCr$^+$, in CD4-depleted mice is consistent with the IFN-γ blocking studies *in vivo* showing that IFN-γ is not the only cytokine involved in the rejection of MMC.

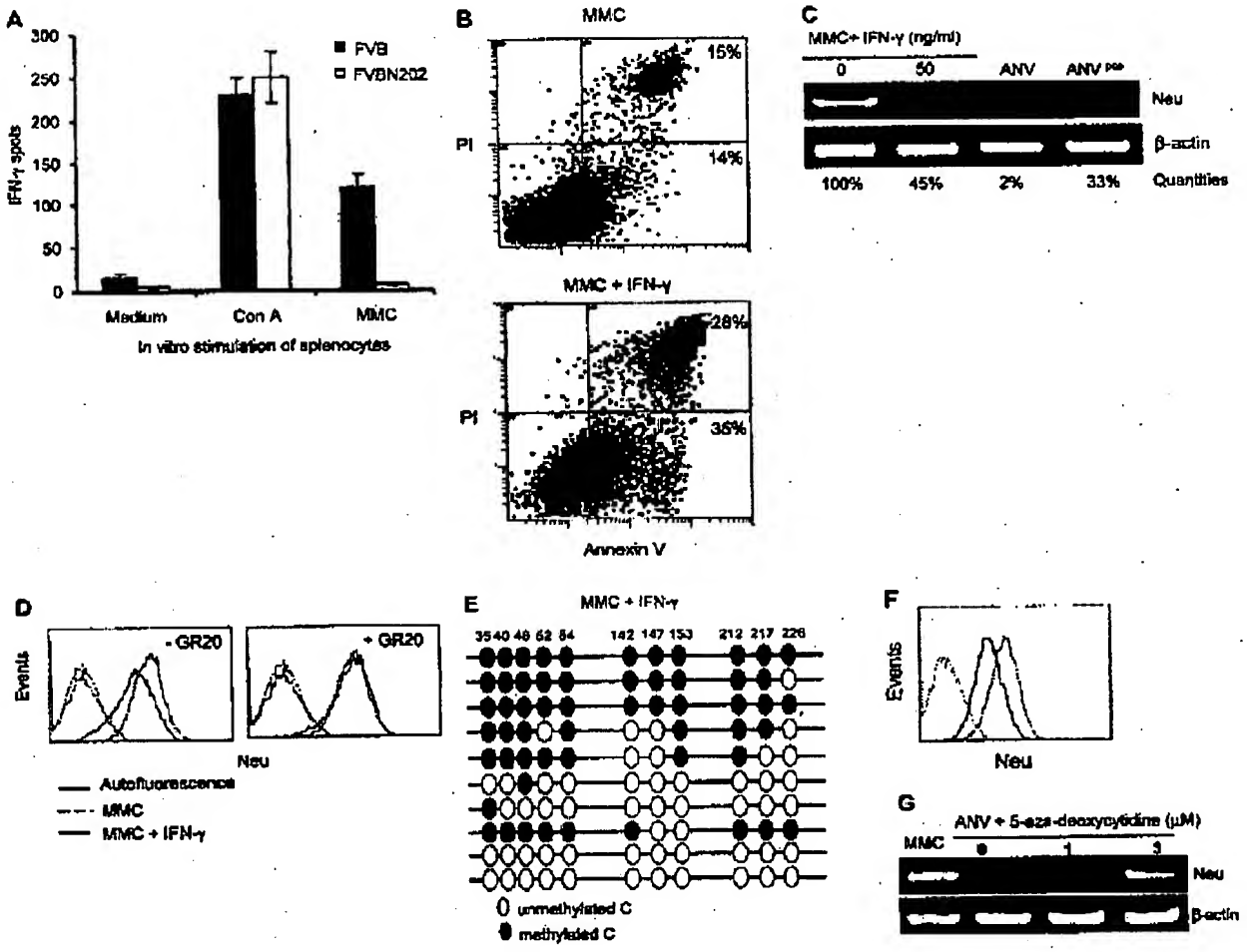

Figure 4. IFN-γ downregulates expression of neu antigen by inducing methylation of the MMTV promoter. (A) Detection of MMC-specific IFN-γ secretion by splenocytes of wild-type FVB or FVBN202-transgenic mice (n=4) 2 wk after challenge with MMC using ELISPOT assay. Splenocytes were stimulated with irradiated MMC *in vitro* at 6:1 ratios. Con A stimulation was used as a positive control. (B) Flow cytometry analyses of early apoptotic (Annexin V-positive) or late apoptotic (PI- and Annexin V-positive) MMC in the absence or presence of IFN-γ for 3 days. (C) Semi-quantitative RT-PCR analysis of neu expression in MMC in the absence or presence of IFN-γ. ANV and ANV^{neg} cell lines were used as controls for loss or intermediate expression of neu mRNA. Neu expression in the PCR products was quantitated in agarose gel using Quantity One 1-D analysis Software. (D) Flow cytometry analysis of the neu expression in MMC in the absence (dashed line) or presence (solid line) of IFN-γ *in vitro*. Left and right panels show *in vitro* culture conditions in the absence or presence of IFN-γ-blocking GR20 Ab (20 μg/mL), respectively. Viable cells were gated in all the FACS analyses. Autofluorescence is shown as dotted lines. Isotype control-induced fluorescence was similar to autofluorescence. (E) Bisulfite genomic sequencing of MMC cells after 3-day treatment with IFN-γ. A total of ten clones per sample was sequenced. (F) MMC were removed from FVB mice at the time of tumor rejection (day 10–14 post-challenge). Expression of the neu protein was detected by flow cytometry either immediately after the MMC removal (solid line) or after 3–4 wk *ex vivo* culture (dashed line). The dotted line indicates autofluorescence. (G) RT-PCR analysis of RNA isolated from untreated MMC or ANV as well as ANV treated with different concentrations of 5-aza-deoxycytidine for 3 days. β-actin was amplified as an internal control.

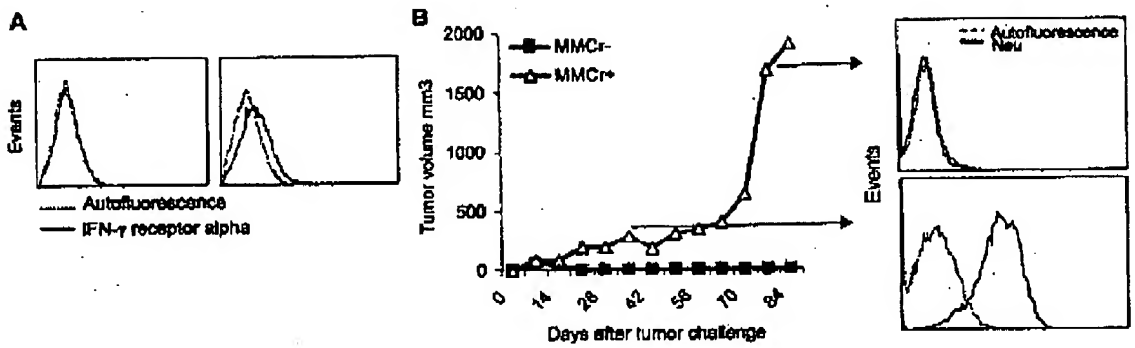

Figure 5. IFN-γ-mediated neu antigen loss and tumor relapse in vivo. (A) Establishment of MMCr$^+$ and MMCr$^-$ clones with the presence or lack of IFN-γ receptor alpha chain obtained from freshly isolated spontaneous mammary tumors or after a number of passages of MMC in vitro, respectively. (B) FVB mice (n=3) were depleted of CD4$^+$ T cells and inoculated with MMCr$^+$ or MMCr$^-$. Tumor growth was monitored until 3 months after the challenge. RT-PCR analysis of neu mRNA expression in the tumors obtained from MMCr$^+$-bearing FVB mice either at the plateau phase of tumor growth (day 40 post-challenge) or at the exponential growth phase (day 85 post-challenge).

Discussion

It was suggested that immunoediting of tumors is facilitated by the genetic instability of tumors [15], particularly in the genes encoding tumor antigens. In order to understand tumor antigen loss during tumor immunoediting, we used the wild-type FVB mouse and the FVBN202-transgenic mouse model of HER-2/neu-positive mammary carcinomas. Unlike wild-type FVB, FVBN202-transgenic mice express rat neu protein under the control of the MMTV promoter, rendering their immune system tolerant to the rat neu oncogene product. Wild-type FVB mice can reject primary MMC in T cell-dependent, neu-specific fashion as shown here with the *in vivo* CD4/CD8 T cell-depletion studies, induction of MMC-specific IFN-γ production, and detection of MMC-specific T cell responses [16]. Since CD4 depletion *in vivo* may also deplete CD4$^+$ NK1.1 cells, we depleted NK1.1 cells *in vivo* to determine whether these cells may participate in MMC rejection. However, the presence of T cells in the absence of NK1.1 cells induced spontaneous rejection of MMC in FVB mice (data not shown). Despite T cell-mediated rejection of MMC, tumor relapse occurred due to neu antigen loss and emergence of ANV. Such neu antigen loss was induced by anti-tumor T cell responses, because expression of neu antigen remained intact in MMC cells derived from T cell-depleted mice. No marked differences were observed in the growth of MMC and ANV when these tumors were inoculated into FVBN202-transgenic mice [12].

It has been suggested that tumor immunoediting occurs due to Darwinian selection, where tumor-specific immune responses eliminate antigen-positive clones, and antigen-negative variants escape and grow [8, 9]. On the other hand, there are reports supporting the hypothesis that anti-tumor immune responses themselves induce changes in antigen-positive clones, converting them into antigen-negative clones [10, 11]. Using a neu-overexpressing primary tumor, MMC, and its relapsed neu-negative variants, ANV, our data in the present studies support the latter.

First of all, we show that neu antigen loss was due to the induction of epigenetic changes in neu-positive MMC in the presence of anti-tumor T cell responses. Initial rejection of MMC in wild-type FVB mice occurred in the presence of MMC-specific IFN-γ-producing T cells. *In vitro* studies showed that IFN-γ was involved in downregulation of neu antigen in MMC. Three-day treatment of neu-overexpressing MMC with IFN-γ downregulated neu expression at both the mRNA level and the protein level in viable MMC. Sensitivity of MMC to IFN-γ-mediated methylation of the MMTV promoter depended on the status of the IFN-γ receptor in MMC. IFN-γ downregulated neu expression in the IFN-γ receptor-positive MMC but not in IFN-γ receptor-negative MMC clones. However, removal of IFN-γ from the culture resulted in normal proliferation of MMC and overexpression of neu after 3 to 4 wk in culture. Interestingly, expression of the neu antigen was downregulated at the time of MMC rejection and recovered after a 3- to 4-wk culture *ex vivo*. Considering that tumor relapse and emergence of ANV occur 1–3 months after the initial tumor rejection, IFN-γ treatment for 3 days might not be long enough to induce complete and/or stable neu loss. Alternatively, other components of the immune response such as neu-specific Ab [17], Fas-Fas ligand interactions, or Granzyme B-mannose-6-phosphate receptor interactions may also contribute to the neu antigen loss. These possibilities remain to be

investigated. It is also likely that MMC clones with low expression of neu antigen may not survive for 3–4 wk after removal of IFN-γ *in vitro*, while neu-positive MMC may survive and become predominant. Although downregulation of the neu protein was detected in MMC at the time of tumor rejection, treatment of animals with GR20 Ab to block IFN-γ receptor *in vivo* did not prevent the rejection of MMC or downregulation of neu expression in wild-type FVB mice (data not shown). This indicates that IFN-γ partially, not solely, contributes in the tumor rejection or induction of epigenetic changes in MMC resulting in downregulation of the neu antigen. Interestingly, IFN-γ receptor-positive MMC clones ($MMCr^+$) relapsed *in vivo* in the presence of $CD8^+$ T cells and absence of $CD4^+$ T cells or anti-neu Ab. Relapsed tumors were ANV. On the other hand, IFN-γ receptor-negative $MMCr^-$ tumors were rejected, and animals remained tumor-free during the trial. These *in vivo* studies suggest that the status of the IFN-γ receptor in tumors will determine rejection or relapse of MMC in FVB mice (manuscript in preparation). It has been shown that loss/downregulation of neu in FVBN202-transgenic mice is controlled by hypermethylation of the MMTV promoter in CpG sites within region 1 [13]. Zhou and coworkers [13] identified ten potential sites of methylation within CpG islands. However, we identified one additional potential site of methylation (position 213) in this region. We detected hypermethylation of the MMTV promoter during antigen loss induced by IFN-γ treatment *in vitro*. ANV cells isolated from animals with intact T cells also showed hypermethylation of MMTV. This further confirmed that IFN-γ-mediated epigenetic changes in MMC result in neu antigen loss and tumor escape. Treatment of ANV with 3 μM of a demethylating agent (5-aza-deoxy-cytidine) was able to reverse neu expression in ANV.

Our findings are consistent with other reports showing that IFN-γ can promote immune-mediated tumor escape by downregulation of gp70 in CT26 tumors [10]. The downstream signaling pathways leading to IFN-γ-mediated antigen loss remain to be determined. Interestingly, expression of HER-2/neu in human breast carcinomas is regulated by the AP-2 transcription factor, which binds a CpG-rich promoter region of the HER-2/neu gene [18]. Therefore, it is likely that methylation of this promoter region induced by IFN-γ may inhibit the expression of HER-2/neu by AP-2. It was reported that treatment of HER-2/neu-positive human ovarian carcinoma cells with IFN-γ reduced the expression of HER-2/neu at both mRNA and protein levels [19]. We also detected IFN-γ-mediated downregulation of HER-2/neu in human breast carcinoma cell lines (manuscript in preparation).

Secondly, we showed that residual neu-negative clones in MMC (MMC^{neg}) and residual neu-positive clones in ANV (ANV^{pos}) differ from ANV and MMC, respectively. ANV^{pos} showed intermediate levels of neu expression as compared to MMC cells, which indicates that ANV^{pos} cells were not MMC left behind following neu-targeted immune responses. The differences between ANV and MMC^{neg} also do not support the hypothesis that ANV might be derived from MMC^{neg} following eradication of neu-positive clones under immune pressure. The heterogenic nature of MMC and ANV allowed us to isolate MMC^{neg} and ANV^{pos} cells and perform comparative studies. The Darwinian selection hypothesis also predicts the presence of such residual clones. These residual neu-negative and neu-positive clones were not artifacts of *in vitro* cell culture, because the status of neu expression in these tumors was validated in several tumor clones, and similar observations were made in fractions of freshly isolated MMC and ANV [16, 17, 20]. In addition, MMC^{neg} and ANV^{pos} were highly stable for the lack of or intermediate expression of neu over 45 passages *in vitro*. Thus, no residual neu-positive clones or neu-negative clones were detected in MMC^{neg} or ANV^{pos}, respectively. Patterns of methylation of the MMTV promoter in four cell lines corresponded to the patterns of surface neu expression in these cell lines. There was one clone in ANV with complete demethylation and two clones in MMC with partial methylation. These clones may represent residual neu-positive and neu-negative clones of ANV and MMC, respectively. The four tumor lines appeared to have distinct gene expression profiles as well as different morphology and proliferation rates. Hundreds of genes were upregulated or downregulated when comparing MMC with ANV, MMC with ANV^{pos}, or ANV with MMC^{neg}. Gene array analyses showed that epithelial markers such as claudin 3 (CLDN3), CLDN4, and occluding (OCLN) [21] were markedly increased in MMC *versus* ANV or MMC *versus* ANV^{pos}, while these genes were not expressed in MMC^{neg}. In addition, mesenchymal markers such as procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide (P4HA1), and snail homolog 1 (SNAI1) as well as actin, alpha 2, smooth muscle, and aorta (ACTA2) [21] were markedly higher in ANV *versus* MMC or ANV^{pos} *versus* MMC. Expression of vimentin (VIM) was higher in ANV than in MMC. These findings are consistent with previous reports [20].

Since microarray analysis showed differentially expressed $H\text{-}2^q$, STAT-1, and Fas in MMC, ANV, MMC^{neg}, and ANV^{pos}, we used semi-quantitative RT-PCR to further validate the microarray findings. These findings in biological replicates of the cells further confirmed differential expression of these molecules in MMC *versus* ANV^{pos} and ANV *versus* MMC^{neg}. These results are consistent with our previous findings showing that MMC and ANV have distinct proteomic profiles [16], and these

tumor lines express comparable levels of IFN-γ receptors, while expression of STAT-1 is downregulated in ANV [14]. This also suggests that ANV should be more resistant than MMC to IFN-γ-induced apoptosis *in vivo*. Others have also reported that relapsed tumors are refractory to IFN-γ-mediated T cell responses [22].

It has been reported that IFN-γ downregulates the NKG2D ligand H60 on tumors, rendering them resistant to NK-mediated killing [23]. Therefore, the epigenetic effect of IFN-γ is not restricted to particular tumor antigens. On the other hand, depending on the antigenic system and mechanism of antigen expression, IFN-γ may or may not modulate particular tumor antigens. Regulatory functions of IFN-γ on the expression of other tumor antigens that are regulated by hypomethylation of the gene promoter remain to be investigated. Our findings are consistent with other reports [10] and further support the hypothesis that IFN-γ simultaneously induces apoptosis and antigen loss in tumors. Therefore, the outcome of anti-tumor immune responses will depend on the balance between these tumor inhibitory and tumor immunoediting effects. IFN-γ is one of the components of anti-tumor immune responses that might be actively involved in neu antigen loss due to the induction of epigenetic changes in primary tumors. Whether other components of the immune response such as Fas ligand, granzyme, or TRAIL contribute to tumor rejection or neu antigen loss also remains to be determined. It has been reported that anti-neu Ab can induce neu antigen loss in mammary carcinomas of FVBN202-transgenic mice [16]. Identification of antigenic epitopes in HER-2/neu that may induce either immune-mediated tumor rejection or tumor relapse would improve peptide-based vaccination approaches to overcome tumor relapse [24, 25].

Materials and methods

Mice

Wild-type FVB (Jackson Laboratories) and FVBN202-transgenic female mice (Charles River Laboratories) were used throughout these studies. FVBN202 is the rat neu-transgenic mouse model in which 100% of females develop spontaneous mammary tumors by 8–10 months of age, with many features similar to human breast cancer. These mice overexpress an unactivated rat *neu* transgene under the regulation of the MMTV promoter [26]. The studies have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University.

Tumor cell lines

The MMC cell line was established from a spontaneous tumor harvested from FVBN202-transgenic mice as previously described, with minor modifications [16, 20]. Tumors were sliced into pieces and treated with 0.25% trypsin at 4°C for 12–16 h. Cells were then incubated at 37°C for 30 min, washed, and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) [27]. The ANV cell line was generated by s.c. inoculation of MMC (5×10^6 to 6×10^6) into the right mid-dorsum of a non-transgenic wild-type FVB mouse. In this mouse, anti-neu immune responses can be elicited, resulting in initial tumor rejection and relapse of ANV after a long latency. MMC^{neg} and ANV^{pos} cell lines were derived from MMC and ANV, respectively, using the Beckman Coulter EPICS Elite sorter. These cells were then cultured in RMPI 1640 supplemented with 10% FBS and were analyzed for the expression of rat neu protein before use. In some experiments, MMC (4×10^6 cells) were cultured in the presence or absence of IFN-γ (50 ng/mL; Serotec, NC).

Flow cytometry

A single staining flow cytometry analysis of the mammary tumor cells (10^6 cells/tube) was carried out using mouse anti-neu (Ab-4) Ab (Calbiochem, San Diego, CA), mouse anti-$H\text{-}2^q$ Ab, isotype control Ig, and FITC-conjugated anti-mouse Ig (BD Pharmingen, San Diego, CA) at the concentrations recommended by the manufacturer. Cells were finally washed, fixed with 1% ultra-pure formaldehyde, and analyzed with the Beckman Coulter EPICS XL within 24 h of fixing. Double staining of viable cells was also performed using Annexin V and propidium iodide (PI) as previously described [28].

ELISPOT assay

Splenocytes of FVB and FVBN202-transgenic mice (inoculated with MMC) were subjected to ELISPOT assay as previously described by our group [12]. Briefly, 96-well filtration plates (Millipore, Bedford, MA) were coated with 10 μg/mL rat anti-mouse IFN-γ Ab (BD PharMingen, San Diego, CA) and subsequently blocked with RPMI 1640 medium containing 10% FBS. RBC were lysed with Tris-NH_4Cl, and 50 μL of the splenocytes (5×10^5 cells/well) were added to each well and incubated with 50 μL Con A (5 μg/mL) or irradiated MMC (15 000 rad; 6:1 E:T ratios) in complete medium (10% FBS, 50 U/mL penicillin/streptomycin, 2 mM L-glutamine, 1 mM 2-ME) at 37°C in an atmosphere of 5% CO_2 for 20–24 h. The plates were then washed extensively and incubated with 5 μg/mL biotinylated anti-mouse IFN-γ Ab (BD PharMingen), followed by a pulse with 0.2 U/mL alkaline phosphatase avidin D (Vector Laboratories, Burlingame, CA). Positive spots were developed by adding 50 μL/well 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium solution (Roche, Indianapolis, IN).

Microarray analysis

The Affymetrix® protocol has been described elsewhere [29, 30]. The GeneChip® Mouse Genome 430A 2.0 array provides comprehensive coverage of the transcribed murine genome by including over 22 600 probe sets that analyze the expression level of over 14 000 murine transcripts. Each chip was scanned at a high resolution, with pixelations ranging from 2.5 μm down to 0.51 μm, with the Affymetrix GeneChip® Scanner

3000 according to the GeneChip® Expression Analysis Technical Manual procedures (Affymetrix, Santa Clara, CA). After scanning, the raw intensities for each probe were stored in electronic files (in .DAT and .CEL formats) by the GeneChip® Operating Software (GCOSv1.1) (Affymetrix). Normalization, background subtraction, and expression values for each probe set were calculated using a method developed by others [31], which is an effective expression summary motivated by a log scale linear additive model. This summary statistics, referred to as the log scale robust multi-array analysis (RMA), uses .CEL files to calculate probe set expression summaries.

Statistical analysis

The microarray analysis was performed with the BRB-ArrayTools v3.1.0 [32], an Excel Add-in that collates microarray data with sample data. Moreover, the "significance-score" algorithm (S-score) was used to produce a score for all the comparisons of the expression summaries between MMC and ANV, MMC and ANV^{pos}, and ANV and MMC^{neg} [33]. The S-score produces a robust measure of expression changes by weighting oligonucleotide pairs according to their signal strength above empirically determined noise levels. The procedure produces scores centered around "0" (no change) with a standard deviation of 1. Thus, scores >2 or <–2 from a single comparison have, on average, a 95% chance of being significant hybridization changes, corresponding to $p<0.05$.

Semi-quantitative RT-PCR

According to the differences detected by microarray analyses, three immunologically relevant genes (neu, STAT-1, and Fas) were selected to validate the differences in gene expression between primary MMC *versus* ANV^{pos} or relapsed ANV *versus* MMC^{neg}. Total RNA (1.5 μg) from four tumor lines was used as templates in a reverse transcription reaction system (total volume of 20 μL). The cDNA were then transferred to a PCR master mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 2.5 U Taq polymerase, and 1 μM gene-specific primers: Neu [5′-ATGATCATCATGGAGCTGGCG-3′ (sense) and 5′-CTAG-GATCTCAGGGTTCTCTGCA-3′ (anti-sense)]; STAT-1 and Fas [34]; and β-actin [5′-GTGGGCCGCTCTAGGCACCAA-3′ (sense) and 5′-CTCTTTGATGTCACGCACGATTTC-3′ (anti-sense)]. PCR conditions were as follows: Neu: 94°C 5 min, 94°C 1 min, 66°C 1 min, 72°C 3 min (40 cycles) followed by 10 min extension at 72°C; STAT-1 and Fas: 94°C 5 min, 94°C 30 s, 60°C 30 s, 72°C 1 min (35 cycles) followed by 5 min extension at 72°C; β-actin: 94°C 5 min, 94°C 30 s, 52°C 30 s, 72°C 1 min (30 cycles) followed by 5 min extension at 72°C. Amplified fragments were visualized by ethidium bromide staining of the agarose gel and photography under UV light in Gel Doc 2000™ (BioRad). Quantity One 1-D analysis Software was used to quantitate each PCR product. Data were normalized using β-actin as an internal control. The highest value in the MMC-positive control was adjusted to 100%, and values for samples were calculated proportionally.

In vivo tumor challenge studies

Female FVB mice ($n=4$) were inoculated s.c. with MMC or ANV (5×10^6 cells/mouse). Animals were inspected twice every week for the development of tumors. Masses were measured with calipers along the two perpendicular diameters. Tumor volume was calculated by: V(volume) = L(length) × $W(width)^2 \div 2$. Mice were killed before the tumor mass exceeded 2000 mm^3. FVBN202-transgenic mice ($n=3$) were also inoculated with MMC or ANV.

Bisulfite genomic sequencing

Genomic DNA was isolated from the tumor cells (6×10^6) using the ZR Genomic DNA Kit (Zymo Research, Orange, CA). For DNA methylation analysis, 0.5 μg DNA was treated with bisulfate using the EZ DNA Methylation Kit according to the manufacturer's protocol (Zymo Research). The reaction was performed using the FastStart High Fidelity PCR System (Roche) and the following primers: forward 5′-GAGAAGTAGT-TAAGGGGTTGTTTTTTAT-3′; reverse 5′-AAATTAACTA-TAATCCTTACCCCAAAAA-3′. PCR reactions were carried out as described previously [13]. The resulting PCR fragments were ligated into the pGEM-T Easy vector (Promega, Madison, WI) and were sequenced.

Demethylation studies

ANV cells were treated with 1 or 3 μM 5-aza-deoxycytidine (EMD Biosciences, San Diego, CA) for 3 days. MMC and ANV were used as positive and negative controls for neu expression. RNA was then isolated and used in a two-step RT-PCR reaction using neu-specific primers that amplified a 474-bp fragment of neu mRNA: sense 5′-AACAGCTCAGAGACCTGCTTTGGA-3′ and anti-sense 5′-TGATCCAAGGACGTTCACGTTCGT-3′. β-ac- was amplified as an internal control.

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